NITRIC OXIDE NEUROMODULATION OF VERTEBRATE GAP
JUNCTION-COUPLED RETINAL HORIZONTAL CELLS

by

Tareq Yousef

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To Mom, Sarah, Kaitlyn, and Patrick.

For the gift I was given for being myself, family.

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ABSTRACT

Horizontal cells are second-order neurons of the vertebrate retina that receive synaptic input from photoreceptors and provide feedback to photoreceptor synaptic terminals or feedforward to bipolar cells. These connections are thought to contribute to the generation of the surround of center-surround receptive fields of bipolar and ganglion cells, important for the detection of contrast. In the retina of goldfish, cone-driven horizontal cell somata (HCS) have axons that terminate as axon terminals (HATs) in the inner nuclear layer (INL) forming contacts with bipolar and amacrine cells. The function of these contacts is unknown. A prominent feature of both HCS and HATs is extensive gap junction coupling, resulting in a large receptive-field size. The coupling between HCS is not static but modulated by the level of ambient illumination, with gap junction coupling decreased following exposure to bright light (light-adaptation) or darkness (dark-suppression) relative to coupling under dim light conditions (light-sensitization). Flickering light also reduces HCS gap junction coupling. Several neuromodulators of gap junction coupling between HCS have been identified. One such neuromodulator, nitric oxide (NO), has been shown to reduce the effect of light-adaptation on HCS coupling. Nitric oxide is thought to promote phosphorylation of gap junction proteins via the action of cyclic GMP and protein kinase G (PKG). Therefore, a primary aim of this thesis was to provide further evidence that NO and PKG are involved in the reduction of horizontal cell gap junction coupling following light-adaptation. "Stab loading" of the gap junction-permeable tracer Neurobiotin was used to study the modulation of coupling between HATs in the goldfish retina. Tracer coupling between HATs showed the same sensitivity to ambient light conditions and application of neuromodulators as reported for HCS, including a role for NO in light-adaptation. Although a PKG inhibitor also reduced the effect of light-adaptation on HAT tracer coupling, so did a PKA inhibitor. PKG inhibitor also reduced the effect of light-adaptation on goldfish HCS receptive-field size (intracellular recording) and tracer coupling ("cut loaded") between mouse HCS. Additional experiments examined dark-suppression and the effect of flickering light on HAT tracer coupling. These light conditions are thought to reduce HCS gap junction coupling via dopamine acting via D1 dopamine receptors, cyclic AMP and PKA. The effect of flickering light was reduced by PKA inhibitor, but not PKG inhibitor, but the effect of dark-suppression was reduced by PKG inhibitor but not PKA inhibitor or NO synthase inhibitor. Taken as a whole, these results suggested that the modulation of HAT gap junction coupling by ambient light is similar to that described previously for HSC. In addition, the findings are generally consistent with an effect of light-adaptation on both HCS and HAT gap junction coupling involving NO and PKG, including some evidence from a mammalian (mouse) retina. The effect of PKA inhibitor on HAT light-adaptation could represent PKG-PKA cross-talk. The results from the study of flickering light on HATs are consistent with the dopaminergic mechanism described previously for HCS. The implied role for PKG in dark-suppression in HATs was unexpected and is not consistent with a mechanism involving dopamine but, given the lack of an effect of NO synthase inhibitor, was also not consistent with a mechanism involving NO. Changes in HCS gap junction coupling could alter the surround of bipolar and ganglion cells generated via feedback, feedforward or possibly via synaptic contacts made by HATs with bipolar cells in the INL.

LIST OF ABBREVIATIONS AND SYMBOLS USED

A/S	Annulus/spot
A-type	Axonless
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BL	Bright light
B-type	Axon-bearing
CBX	Carbenoxolone
CO	Carbon monoxide
CTRL	Control
Cx	Connexin
DA	Dopamine
DAergic	Dopaminergic
dbcAMP	Dibutyryl cyclic AMP
dbcGMP	Dibutyryl cyclic GMP
DMSO	Dimethyl sulfoxide
DL	Dim light
dLGN	Dorsolateral geniculate nucleus
DS	Dark sensitization
FL	Flickering light
GCL	Ganglion cell layer
GMP	Guanosine monophosphate
HAT	Horizontal cell axon terminal
HCS	Horizontal cell somata
I_0	Light irradiance
INL	Inner nuclear layer
IPC	Interplexiform cell

IPL	Inner plexiform layer
LA	Light-adaptation
LS	Light-sensitization
MFA	Meclofenamic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
OPL	Outer plexiform layer
PDE	Phosphodiesterase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
RA	Retinoic acid
R_i	Internal resistance
R_m	Membrane resistance
RPE	Retinal pigmented epithelium
SMTC	S-methyl-thiocitrulline
TPA	Phorbol 12-tetradecanoil 13-acetate

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CHAPTER 1:
INTRODUCTION

1.1 Preamble

The vertebrate retina is a thin sheet of central nervous tissue that lines the back of the eye and contains neurons that convert the image of the visual world into an electrochemical signal interpreted by the brain. Much has been learned about the neuronal elements that make up the retina and several excellent textbooks and review articles describe the current state of knowledge about the arrangement and interactions of retinal neurons (Dowling, 1987; Demb and Singer, 2015). Visual pigment-containing photoreceptors, rods and cones, transduce photons incident on the retina into changes in membrane potential. Rods are more sensitive to light than cones, operating under dim light conditions (e.g., at night). Cones require more light to respond, (contributing to vision during the day) and there can be multiple subtypes that contain different visual pigments, representing the initial steps required for colour vision. The change in photoreceptor synaptic terminal membrane potential governs the release of the neurotransmitter glutamate onto the processes of second-order neurons, bipolar and horizontal cells, at the level of the outer plexiform layer (OPL) (see Fig. 1.1 and Fig. 1.2). Bipolar cell axon terminals make glutamatergic synapses with amacrine and ganglion cell dendrites within the inner plexiform (IPL) and ganglion cell layer (GCL). The light-evoked responses of most of these neurons are graded changes in membrane potential, but retinal ganglion cells give rise to long axons that form the optic nerve and signal their targets in the brain via action potentials.

The basic connectivity described above applies to the cone pathway of most vertebrates, but the rod pathway is different in mammals. Like cones, rods provide synaptic input to bipolar cells, but the rod-connected bipolar cells do not contact ganglion

cells directly. Instead, the signal is passed via an amacrine cell (the rod amacrine cell otherwise known as the AII amacrine cell) to cone-connected bipolar cells that, in turn, synapse with ganglion cells (Fain and Sampath, 2018; Lauritzen et al., 2019).

A fundamental feature of visual processing in all vertebrate retinas is the creation of center-surround receptive-fields in the retina. Ganglion cells were the first retinal neurons that were shown to possess such receptive fields (Kuffler, 1953) but, as will be described further below, it is also a property of bipolar cells. Center-surround receptive fields are characterized by having different responses depending on what portion of the total receptive field is stimulated. For example, for certain ganglion cells, stimulating the central part of the receptive field (e.g., with a small spot) will result in an increase in action potential firing rate but if the periphery of the receptive field is stimulated (e.g., with an annulus) the firing rate will decrease. Such a cell is described as an ON-center, OFF-surround ganglion cell. There is another broad type of ganglion cell response, one where stimulation of the center decreases firing rate and stimulation of the periphery increases activity. These are termed OFF-center, ON-surround ganglion cells. If, in either type of ganglion cell, both the center and surround are stimulated, the response is weak or even absent, with the stimulation of both components of the receptive field (center and surround) cancelling each other out.

The utility of center-surround receptive organization is that it allows the visual system to respond not to the absolute level of illumination within each ganglion cell receptive field but only when there is a difference (called contrast) between one (smaller) part of the receptive field (the center) and the larger background (the surround). In fact, contrast borders/edges are probably the natural stimuli that drive center-surround

receptive fields the most (spots and annuli are not all that common in natural scenes), a result of a contrast edge falling along the border of the center and surround component of the receptive field allowing stimulation of the center but less stimultion of the surround (Fig. 1.3). The two types of ganglion cells (ON-center, OFF-surround; OFF-center, ON-surround) allow sensitivity to the full range of possible contrasts: from objects brighter than the background (exciting ON-center cells, inhibiting OFF-center cells) to the opposite, objects dimmer than the background (exciting OFF-center cells, inhibiting ON-center cells).

1.2 Circuitry that generates center-surround receptive-fields

The standard view of the circuitry generating center-surround receptive fields indicates a key role for bipolar cells (Kaneko, 1973; Dacey et al., 2000; Fahey and Burkhardt, 2003). First, two broad types of bipolar cells are responsible for the ON and OFF pathways. The difference is mediated by the action of glutamate released from photoreceptors acting on two different types of receptors on bipolar cell dendrites. ON bipolar cells depolarize to light, an effect mediated by metabotropic (mGluR6) glutamate receptors. OFF bipolar cells hyperpolarize to light, an effect mediated by ionotropic (AMPA) glutamate receptors. The synapses made by bipolar cell axon terminals onto ganglion cell dendrites drive the responses of ganglion cells when bipolar cells depolarize and glutamate is released.

The surround component of the bipolar cell response is thought to be generated by horizontal cells and amacrine cells through lateral interactions within the retina. Here we shall limit our consideration to the role of horizontal cells. Horizontal cells receive synaptic input from photoreceptors via ionotropic (AMPA) glutamate receptors and

hyperpolarize in response to full-spectrum (white) light. There are two features of horizontal cells that are important for the generation of the receptive-field surround of bipolar cells: feedback to photoreceptors and gap junction coupling.

Horizontal cells not only receive synaptic input via processes in the OPL, but the processes are also pre-synaptic, influencing the membrane potential of the photoreceptor terminal via negative feedback (Murakami et al., 1982; Fahrenfort et al., 2005; Thoreson and Mangel, 2012). The term "negative" indicates that the influence of horizontal cells on photoreceptor terminals is opposite (depolarizing) to the effect of light on the photoreceptor (hyperpolarizing). It is this interaction between horizontal cells and photoreceptor terminals that is thought to be at the heart of the generation of the receptive field surround.

Though it is known that horizontal cells respond to light with graded hyperpolarizations, the specific mechanism by which feedback is generated has been a topic of wide debate (Thoreson and Mangel, 2012; Kramer and Davenport, 2015). The three leading hypotheses, namely GABA release, alkalinization of the synaptic cleft, and an ephaptic mechanism, all have in common that feedback increases calcium channel currents in photoreceptor terminals (Verweij et al., 1996; Hirasawa and Kaneko, 2003; Verweij et al., 2003; Vessey et al., 2005; Cadetti and Thoreson, 2006). The debate about the mechanism was driven partly by the implicit assumption that there could be only one mechanism, or that perhaps more than one mechanism operated in parallel. However, it has been recently suggested that two of the mechanisms may be linked, with horizontal cell vesicular GABA release mediating increased HCO_3^- efflux thereby increasing synaptic pH in response to light (Barnes et al., 2020; Hirano et al., 2020).

The mechanism mediating horizontal cell to photoreceptor feedback does not explain the spatial feature of the surround, that it forms a large region peripheral to the receptive-field center. This is explained partly by the 'horizontal' orientation of horizontal cells within the retina and the extensive coupling of horizontal cells by gap junctions.

1.3 Gap Junctions

Gap junctions are trans-membrane proteins that form channels through two adjacent plasma membranes. The first evidence of gap junctions came from electron microscope images of cardiomyocytes where the plasma membrane of adjacent cells came into close apposition (Sjöstrand et al., 1958). Similar regions of close apposition were soon discovered in Mauthner cells of the goldfish brain (Robertson, 1963). But it was Revel and Karnovsky (1967) that emphasized the 20 nm "gap" separating plasma membranes at the areas of apposition and this was the origin of the term "gap junction" (Robertson, 1963; Wells and Bonetta, 2005).

It was ultimately shown that within the regions of apposition are proteins made up of individual units that were called connexins, the first one described being connexin 43 (Cx43) from heart tissue (Beyer et al., 1987). Six connexins form a hemichannel, or connexon, that connects the cytoplasm of one cell to that of another cell via another hemichannel (Bruzzone et al., 1996). The joining of two connexons forms a functional gap junction channel that acts as a conduit for small molecules, ions and electrical current and, hence, are often also called electrical synapses.

There are numerous types of connexins, derived from ~20 genes, with widespread distribution in tissues. Gap junctions are abundant in the retina, with the connexin

composition depending on which neurons are involved (for review see (Völgyi et al., 2013)). For example, in the mammalian retina photoreceptor, amacrine cell and ganglion cell gap junctions are formed by Cx36 and horizontal cell gap junctions by Cx50 and Cx57. In some cases, one type of connexin, in one cell, will couple with a different connexin type in another cell, forming a heterotypic junction. For example, gap junction coupling between the rod (AII) amacrine cell and ON cone bipolar cells is mediated by both homotypic (Cx36/Cx36) and heterotypic (Cx36/Cx45) junctions. Additional connexin genes have been found in non-mammalian species and several connexins (e.g. Cx49.5, Cx52.6, Cx53.8, Cx55.5) are associated with horizontal cells in the carp (*Cyprinus carpio*) (Greb et al., 2017).

Gap junction conductance can be modulated by voltage, intracellular pH or Ca^{2+} (Völgyi et al., 2013), but it is not clear that any of these control gap junction coupling between retinal neurons under normal conditions. However, most connexins contain intracellular phosphorylation sites accessible to protein kinases (Lampe and Lau, 2000) that then modulate gap junction coupling (Warn-Cramer and Lau, 2004; Moreno and Lau, 2007). Protein kinases catalyze the transfer of γ-phosphate from adenosine triphosphate (ATP) to the hydroxyl group of phosphorylation site amino acids of target proteins (Lampe and Lau, 2000) with dephosphorylation controlled by phosphatase enzymes (Lochner and Moolman, 2006). Phosphorylation by protein kinases makes possible several means by which gap junctions can be modulated, the result produced by the action of various neuromodulators and their resulting intracellular cascades.

1.4 Horizontal cells contribute to the receptive-field surround

Horizontal cells are coupled extensively by gap junctions (see Fig. 1.2) resulting in a receptive-field size much larger (on the order of millimetres) than their physical size (10-100 µm) (Yamada and Ishikawa, 1965; Naka and Rushton, 1967; Packer and Dacey, 2002). The large receptive-field size of horizontal cells is consistent with their role as contributors to the receptive field surround of bipolar cells. In fact, there is considerable evidence that horizontal cells are an important source of the surround.

Current injection-induced hyperpolarization in horizontal cells (mimicking the effect of light) has been shown to produce surround-like responses in bipolar cells in a variety of species. In the turtle, hyperpolarizing current injection into a horizontal cell produced hyperpolarizing responses in ON-center bipolar cells and depolarizing responses in OFF-center bipolar cells (Marchiafava, 1978) and similar results were obtained in teleost fish (specifically *Cyprinus carpio*) (Toyoda and Tonosaki, 1978a; Toyoda and Tonosaki, 1978b; Toyoda and Kujiraoka, 1982). In the channel catfish (*Ictalurus punctatus*), hyperpolarizing current injection into a horizontal cell affected ganglion cell discharge, mimicking surround responses (Naka and Nye, 1971). Similar evidence has been reported in studies of ganglion cells in the dogfish (*Mustelus canis*) (Naka and Witkovsky, 1972) and the pigmented rabbit retina (Mangel and Miller, 1987; Mangel, 1991).

The preceding has described a model that puts the bipolar cell as the focus of the generation of center-surround receptive fields. The center component is generated by the "vertical" pathway of the retina, from photoreceptors to bipolar cells to ganglion cells. The surround is contributed, at least in part, by horizontal cells via negative feedback to

photoreceptors modulating the input to bipolar cells with a spatial profile endowed by extensive gap junction coupling. It has been suggested that horizontal cells may also make direct ("feedforward") contacts with bipolar cells, contributing a third of the surround response (Yang and Wu, 1991). The mechanism of such a feedforward pathway is not clear. However, a recent study in mouse suggested that horizontal cells make both feedback projections to cones and feedforward contacts to bipolar cell dendrites both at the level of the photoreceptor synaptic terminal (Behrens et al., 2022). Although horizontal cells contribute to the surround of bipolar cells, the surround component of ganglion cells is likely also influenced by amacrine cells acting at bipolar cell to ganglion cell synapses in the IPL (Kolb, 1997, 2007).

1.5 Horizontal cell types and responses

The contribution of horizontal cells to retinal circuit, described above, is likely a feature of all vertebrate retinas. There are, however, some important differences of horizontal cell connectivity in different species (Kaneko and Yamada, 1972; Tsukamoto et al., 1987). In mammals, horizontal cells are categorized into axonless (A-type) or axon-bearing (B-type) cells. Both A- and B-type cell dendrites contact cones. B-type cell axon terminals contact rods. In teleosts, in which horizontal cells have been studied extensively, horizontal cells with processes that contact cones all contain an axon. But unlike mammals, these axons do not make contact with photoreceptors but rather descend into the middle part of the inner nuclear layer (INL) forming horizontal cell axon terminals (HATs) that may make synaptic contacts with amacrine, interplexiform and bipolar cells (See Fig 1.2) (Dowling et al., 1966; Marc and Liu, 1984; Marshak and Dowling, 1987). The neurochemical identity of HAT synapses is unknown and the

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function of the HAT contacts within the INL is not understood. Interestingly, HATs are also coupled by gap junctions (Kaneko and Stuart, 1984) (Fig. 1.2) mediated, at least partially, by Cx53.8 and Cx55.5 (Greb et al., 2017) and have a large receptive-field size (Kaneko, 1970; Shigematsu and Yamada, 1988). In teleosts, a single type of axonless horizontal cell makes contact with rods.

In many vertebrates, horizontal cells respond to light with graded hyperpolarizations regardless of wavelength. However, in some cases, such as fishes (including the families *Serranidae, Mugilidae, Lutianidae, Gerridae,* and *Centropomidae*), there can be multiple types of cone-connected horizontal cells with responses that depend on wavelength (MacNichol Jr and Svaetichin, 1958). For example, in goldfish (*Carassius auratus*), one type (H1) hyperpolarizes regardless of wavelength, but H2 cells hyperpolarize to short and middle wavelengths but depolarize to long wavelengths and H3 cells hyperpolarize to short and long wavelengths but depolarize to middle wavelengths. These differential chromatic responses are thought to contribute to color vision in fish (including *Cyprinus carpio*), by adding wavelength features to the center-surround receptive fields of bipolar and ganglion cells (Kaneko, 1973; Kaneko and Tachibana, 1983).

As described above, horizontal cell responses to light are generally characterized as graded changes in membrane potential, reflecting changing release of glutamate from photoreceptor terminals. However, it has been suggested that, under certain conditions, horizontal cells can produce action potentials. In fact, spontaneous action potentials have been recorded from isolated teleost horizontal cells or in slice preparations and have been shown to be due to Ca^{2+} influx (Tachibana, 1981; Shingai and Christensen, 1986;

Country et al., 2021). How Ca^{2+} action potentials contribute to retinal processing is not clear, especially given the slow time course of the graded potentials that dominate the outer retina.

1.6 Horizontal cell gap junction coupling is dynamic

The role of horizontal cells in retinal processing is made all the more interesting by the fact that horizontal cell gap junction coupling (and horizontal cell receptive-field size) is not fixed but is modulated by the level of ambient illumination (Baldridge, 2001). Much of the evidence of horizontal cell gap junction modulation was obtained using isolated teleost retina preparations and this continues to be a useful system in which to study horizontal cell coupling.

Intracellular recordings of hybrid bass (*Morone chrysops* and *Morone saxatilis*) horizontal cells, obtained from retinas subjected to moderate levels of illumination, had large amplitude responses and large receptive fields (Baldridge et al., 1995). That the large receptive-field size was due to gap junction coupling was revealed by the spread of gap-junction permeable dye (Lucifer yellow) after intracellular injection (Baldridge, 2001). When isolated goldfish (*Carassius auratus*) retinas were exposed to periods of bright background illumination, there was some decrease in the responsiveness of horizontal cells but a clear reduction of receptive-field size (Baldridge and Ball, 1991). Under these conditions, the spread of dye was reduced, indicating that the reduction of receptive-field size was due, at least in part, to reduced gap junction coupling (Baldridge, 2001). In contrast, if isolated retinas (including white perch *Roccus americana*, hybrid bass, and carp *Cyprinus carpio*) were maintained in complete darkness for a prolonged period of time (≥ 1 hr) this resulted in a great reduction of response amplitude of

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horizontal cells, reduction of receptive-field size and decreased gap junction coupling (Knapp and Dowling, 1987; Baldridge et al., 1995; Weiler et al., 1997).

The alteration of horizontal cell responsiveness, receptive-field size, and gap junction coupling by different illumination conditions led to the hypothesis of triphasic adaptation (Baldridge, 2001). Horizontal cells from retinas subjected to prolonged darkness were described as being "dark-suppressed," highlighting the reduction of response amplitudes. Horizontal cells subjected to moderate illumination were termed "light-sensitized," because of the larger (relative to dark-suppressed) response amplitude. Horizontal cells exposed to bright light, with some reduction in responsiveness but a clear reduction in receptive-field size and gap junction coupling, were termed "light-adapted" (Table 1.1). The term light adaptation is better known as the intrinsic adjustment of photoreceptor response gain with changes in ambient illumination. Here, it refers specifically to the effect of steady bright illumination on horizontal cell gap junction coupling that is hypothesized to be mediated by nitric oxide signalling.

The triphasic change of horizontal cell coupling is not unique to the teleost retina. A similar phenomenon was reported for A- and B-type horizontal cells in the rabbit retina (Bloomfield et al., 1997) and others reported similar changes of horizontal cell responsiveness in goldfish (*Carassius auratus*) and again in rabbit (Wang and Mangel, 1996; Hanitzsch and Bligh, 1998).

1.7 Impact of triphasic adaptation on bipolar and ganglion cell receptive fields

If, as described above, horizontal cells contribute to the generation of the receptive-field surround of bipolar and ganglion cells, what would be the impact of changes of

horizontal cell receptive-field size and responsiveness during different components of triphasic adaptation? As described above, horizontal cell gap junction coupling, and receptive-field size are reduced following prolonged darkness. But equally important, the responses of horizontal cells are strongly suppressed (hence the term, dark-suppression). This is important because decreased gap junction coupling would result in an increase in the input resistance (Pereda et al., 2013) that could increase the amplitude of horizontal cell responses to light. Therefore, the combined decrease of horizontal cell gap junction coupling and responsiveness following prolonged darkness could decrease both the size and strength of the receptive-field surround of bipolar and ganglion cells. Interestingly, in cat (Barlow et al., 1957; Rodieck and Stone, 1965; Maffei et al., 1971; Yoon, 1972; Enroth-Cugell and Lennie, 1975; Barlow and Levick, 1976; Peichl and Wässle, 1983), frog (Donner and Reuter, 1965) and rabbit (Masland and Ames 3rd, 1976; Jensen, 1991; Muller and Dacheux, 1997), the strength of the receptive-field surround weakens when ganglion cells are recorded after periods of darkness. The reduction, or even loss, of the receptive-field surround would increase the sensitivity of ganglion cells to dim light by removing the inhibitory influence of the surround. This would favour the sensitivity to light at the expense of contrast detection.

There are no studies that explicitly compared the receptive-field surround of bipolar or ganglion cells in retinas exposed to bright adapting light (light-adaptation). One can only speculate about the utility of uncoupling horizontal cells with increasing levels of illumination. It is possible that changes of receptive-field size during light-adaptation reduce the size of the receptive-field surround allowing for center-surround antagonism

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on a finer scale. This could allow for differential levels of center-surround antagonism depending on the level of ambient light present in different parts of the visual scene.

1.8 Neuromodulation of horizontal cell gap junction coupling

Three neuromodulators that affect horizontal cell gap junction coupling have been identified: dopamine, retinoic acid and nitric oxide (NO). In each case, the action of the neuromodulator was studied by applying test drugs to retina preparations that would be considered "light-sensitized." That is, prior to treatment the horizontal cells had large amplitude responses, large receptive-field sizes and increased gap junction coupling.

1.8.1 Dopamine

Dopamine was the first neuromodulator that was shown to reduce horizontal cell receptive-field size and decrease gap junction coupling (Teranishi et al., 1983; Piccolino et al., 1984; He et al., 2000). Dopamine acts on horizontal cells via D1 dopamine receptors (Schorderet and Nowak, 1990; Nguyen-Legros et al., 1999) leading to activation of adenylyl cyclase, increased cyclic adenosine monophosphate (cyclic AMP) and activation of protein kinase A (PKA) (Lasater, 1987; McMahon et al., 1989; Dowling, 1991; Roy and Field, 2019) that then is thought to phosphorylate horizontal cell connexins, reducing channel conductance. There is, as yet, no study reporting the phosphorylation of a definitive horizontal cell connexin, but fish (bass) Cx35, and the mammalian homologue Cx36, have been shown to be phosphorylated by PKA (Urschel et al., 2006).

In many vertebrate retinas, dopamine is produced by and released from dopaminergic amacrine cells in the INL (Jeon et al., 1998). Therefore, to reach horizontal cells requires passive diffusion through the extracellular space (Teranishi et al., 1983).

However, in New World Primates, humans, cats and teleost fish, dopamine is produced by interplexiform cells (Kolb, 1997). Dopaminergic interplexiform cells also make contacts within the IPL but, unlike dopaminergic amacrine cells, they also send neurites to the OPL, releasing dopamine within proximity to photoreceptors and horizontal cells (Frederick et al., 1982; Yazulla and Zucker, 1988; Kolb et al., 1990).

1.8.2 Retinoic Acid

Retinoic acid, an important morphogen during vertebrate development and a metabolite of vitamin A, is synthesized from retinaldehyde by dehydrogenases located in the retinal pigmented epithelium (RPE), Müller cells (radial glia of the retina) and some amacrine cells (Edwards et al., 1992; Milam et al., 1997). Prompted by the possibility that retinoic acid levels increase in the vertebrate retina during light (McCaffery et al., 1996), it was demonstrated that all-*trans*-retinoic acid reduces teleost horizontal cell receptive-field size and uncouples horizontal cell gap junctions, as evidenced by reduced spread of Lucifer yellow (Weiler et al., 1998; Pottek and Weiler, 2000). The mechanism by which retinoic acid might act on horizontal cell gap junctions is not clear. Although there is evidence that retinoic acid can lead to the activation of protein kinase C (PKC) (Kurie et al., 1993; Radominska-Pandya et al., 2000), the PKC activator phorbol 12-tetradecanoil 13-acetate (TPA) had no effect on gap junction currents recorded from isolated teleost horizontal cells (Zhang and McMahon, 2000). In fact, it has been suggested that retinoic acid acts through external retinoic acid receptor-like binding sites associated with gap junctions but with an action independent of second messengers (Zhang and McMahon, 2000).

1.8.3 Nitric oxide

Nitric oxide (NO) is a gas synthesized by the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS) (Wiesinger, 2001). There are three types of NOS, with neuronal NOS (nNOS) as the isoform that dominates in the nervous system (Bredt et al., 1990; Dawson et al., 1991). nNOS is regulated by the intracellular free calcium concentration ($[Ca^{2+}]_i$) and the Ca^{2+}-binding protein calmodulin (Schmidt et al., 1992). Taken as a whole, studies of vertebrate retinas have collectively localized nNOS to every class of retinal neuron (Villani and Guarnieri, 1996). Studies of nNOS, detected by immunohistochemistry or *in situ* hybridization, suggest photoreceptor inner segments and certain types of amacrine cells are the most common nNOS-expressing neurons in the retina (Vielma et al., 2012). Consistent with this is evidence that levels of NO, assessed by NO-sensitive DAF-2 fluorescence imaging, is the greatest in these same two retinal neuron types (Blute et al., 2000; Giove et al., 2009). This has led to a hypothesis (Vielma et al., 2012) that NO is produced at the level of photoreceptors, possibly under the control of the direct effect of light on photoreceptors, and in the inner retina by amacrine cells under the control of synaptic input from bipolar cells. In fact, recent evidence in mouse suggests that a network of gap junction-coupled amacrine cells are the dominant source of NO in the inner retina (Jacoby et al., 2018).

Nitric oxide is membrane permeable and, therefore could potentially affect multiple targets once synthesized. However, a major limitation is that the effective distance of NO can be limited greatly by enzymatic and chemical consumption of NO within tissues (Hall and Garthwaite, 2009) resulting in mostly local action of NO, within or near the cells producing it. It has been suggested that this means that the action of NO synthesized by amacrine cells is limited to the inner retina and that NO produced by

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photoreceptors may only affect photoreceptors themselves (Vielma et al., 2012). On the other hand, there is in fact evidence, both in lower vertebrate and mammalian retina, suggesting that horizontal cells and bipolar cells contain nNOS and, more importantly, nNOS has also been localized to the OPL (Liepe et al., 1994; Haverkamp and Eldred, 1998; Cao and Eldred, 2001). These sources of NO would be in a better position to target horizontal cells than photoreceptors or amacrine cells. Consistent with this is evidence from DAF-2 imaging that horizontal cells produce NO (Eldred and Blute, 2005).

Several studies have shown that NO, applied to retina using molecules that spontaneously release or, subject to metabolism, produce NO (NO donors), reduces horizontal cell receptive-field size and/or gap junction coupling (DeVries and Schwartz, 1989; Pottek et al., 1997; Xin and Bloomfield, 2000; Daniels and Baldridge, 2011). A common pathway for the action of NO is the activation of soluble guanylyl cyclase leading to elevated cyclic guanosine monophoshate (GMP) and activation of protein kinase G (PKG) (Garthwaite, 1991). Consistent with such a mechanism cyclic GMP (or analogues) have also been shown to reduce horizontal cell coupling (Lu and McMahon, 1997; Xin and Bloomfield, 2000). It has not yet been demonstrated that a definitive horizontal cell gap junction connexin is phosphorylated by PKG but PKG has been shown to phosphorylate Cx35 (Patel et al., 2006).

1.9 Neuromodulators and horizontal cell responsiveness

The focus of the neuromodulator action discussed above are the effects and mechanisms that reduce horizontal cell gap junction coupling. There is also evidence that these neuromodulators can affect horizontal cell responsiveness.

When applied to the retina, dopamine produces a depolarization of the dark-resting membrane potential of horizontal cells and a large ($\geq$50%) reduction of the amplitude of responses to full-field (illumination of the entire retina) stimuli (Mangel and Dowling, 1987; Yang et al., 1988b). Under conditions of full-field stimuli all horizontal cells are affected equally by the changes in transmitter release from photoreceptors. Therefore, changes in gap junction coupling between horizontal cells would have no effect on the responses of horizontal cells to full-field stimuli (Usui et al., 1983). The reduction of horizontal cell responsiveness, and the depolarization of the dark-resting membrane potential, are believed to be due to the enhancement of horizontal cell glutamate receptors by increased cyclic AMP (cyclic AMP) mediated by D1 dopamine receptors (Knapp et al., 1990) that activate PKA (Liman et al., 1989).

In contrast to the action of dopamine, retinoic acid slightly increases horizontal cell responsiveness (Pottek and Weiler, 2000), but the mechanism of action has not been explored. NO also has been reported to increase slightly the responsiveness of horizontal cells and similar results were obtained using cyclic GMP (Pottek et al., 1997; Xin and Bloomfield, 2000).

1.10 Neuromodulators and triphasic adaptation

The preceding sections have established that horizontal cell responsiveness, receptive-field size, and gap junction coupling can be modulated by the level and duration of prior illumination (triphasic adaptation) or by drugs activating pathways associated with dopamine, retinoic acid or NO. The obvious question that follows is: do any of the neuromodulators act endogenously to mediate horizontal cells under the conditions of dark-suppression or light-adaptation?

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In each case, a starting point were suggestions that the level of each of these neuromodulators is increased in the retina by light and, therefore, one or more of them could be mediators of light-adaptation. Evidence from many vertebrates, such as in the clawed frog (Boatright et al., 1994); primates, including humans (Boelen et al., 1998); mouse (Cameron et al., 2018; Perez-Fernandez et al., 2019) and teleost (Weiler et al., 1997) reported that dopamine levels increased upon exposure to light. Light exposure has also been reported to increase levels of retinoic acid in mouse retina (McCaffery et al., 1996; Weiler et al., 2000) and the release of NO (Neal et al., 1998; Djamgoz et al., 2000). In each case these experiments were done using retina preparations where neuromodulators were measured from superfusate solution.

Measurements of neuromodulator release imply a role in the modulation of horizontal cells but this does not necessarily indicate definitively that this is the case. The measurement of neuromodulator release into superfusate does not necessarily indicate that such release occurs at the level of the horizontal cells. Furthermore, it is difficult to know to what extent overflow of neuromodulator into superfusate represents the level present within microdomains of the retina, at or distant from the release sites.

If it is the case that each of the neuromodulators is an important contributor to light-induced uncoupling of horizontal cells (light-adaptation), it would be predicted that drugs that block their action should also limit or block horizontal cell light-adaptation. This, in fact, turned out not to be the case for dopamine: D1 dopamine receptor antagonists, or ablation of dopaminergic neurons in the retina by prior treatment with the neurotoxin 6-hydroxydopamine, did not block or reduce horizontal cell light-adaptation in the teleost retina (Baldridge and Ball, 1991; Umino et al., 1991). At the time these experiments

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were done, dopamine was the only known modulator of horizontal cell coupling. It therefore came as a surprise that dopamine was not responsible for the light-induced reduction of horizontal cell receptive-field size and gap junction coupling.

The reduction of horizontal cell gap junction coupling by light (light-adaptation), as described above, employed exposure to steady light. Flickering light was also found to reduce horizontal cell receptive-field size and gap junction coupling and this effect was blocked by concurrent treatment with the dopamine receptor antagonist haloperidol (Umino et al., 1991). These results suggest that flickering light reduces horizontal cell gap junction coupling and that this, unlike the effects of steady light (light-adaption), is mediated by dopamine. Flickering light has also been shown to produce an even greater release of dopamine from teleost retina than steady light (Weiler et al., 1997) and similar results have been found in other vertebrate retinas (e.g., cat) (Kramer, 1971).

It has also been suggested that dopamine may play a role in the reduction of horizontal cell responsiveness in retinas kept in darkness for a prolonged period (dark-suppression). Yang et al. (1988b) demonstrated that the reduction of horizontal cell responsiveness following prolonged darkness did not occur in retinas treated with the D1-dopamine receptor antagonist SCH-23390 or in retinas where the endogenous source of dopamine was destroyed by prior treatment with 6-hydroxydopamine. Because prolonged darkness also leads to reduced horizontal cell gap junction coupling, it was concluded that this too is mediated by dopamine (Tornqvist et al., 1988). There is also some evidence of increased dopamine release following prolonged darkness (Weiler et al., 1997).

It is worth noting that horizontal cell gap junction coupling does not appear to be influenced by an endogenous (circadian) clock (Ribelayga and Mangel, 2003). This result was surprising because other retinal functions suggested clock-driven modulation involving dopamine (Mangel, 2001). However, the clock-driven effects are all mediated by D2 dopamine receptors that are 2 to 3 orders of magnitude more sensitive to dopamine than D1 dopamine receptors (Hillman et al., 1995). Consequently, Ribelayga and Mangel (2003) proposed that the levels of of dopamine in the retina controlled by the circadian clock is less than that produced by light conditions, adequate to affect D2 dopamine receptors but below the threshold for D1 dopamine receptor-mediated effects, namely horizontal cell gap junction coupling.

When applied to light-sensitized retinas, retinoic acid produces the same result as exposure to bright light (light-adaptation): reduced horizontal cell receptive-field size and gap junction coupling (Weiler et al., 1998; Pottek and Weiler, 2000). This does not prove that retinoic acid is a light-induced endogenous neuromodulator acting on horizontal cells. Unfortunately, no drugs that selectively block retinoic acid are available, making it difficult to assess the contribution of retinoic acid to horizontal cell light-adaptation.

Treatment with NO donors also influences horizontal cells similar to light-adaptation DeVries and Schwartz, 1989; Pottek et al., 1997; Xin and Bloomfield, 2000; Daniels and Baldridge, 2011). In addition, Daniels and Baldridge (2011) reported that the effect of exposure to bright light (light-adaptation) on horizontal cell receptive-field size was reduced during treatment with the NO synthase inhibitor, L-NAME. However,

they did not confirm that L-NAME blocked the reduction of gap junction coupling using dye or tracer coupling (see Section 1.12).

In summary, there is evidence that the reduction of horizontal cell receptive-field size and gap junction coupling produced by prolonged darkness (dark-suppression) or flickering light is mediated, at least partially, by dopamine. Dopamine does not appear to be involved in the effect of steady bright light (light-adaptation) but there is evidence that NO is involved. It remains to be shown definitively that NO is responsible for reduced horizontal cell gap junction coupling following light-adaptation. The role of retinoic acid remains unclear given the absence of specific drugs and uncertainty about the mechanism of action.

1.11 Protein kinases and horizontal cell gap junction coupling

As described above, a common mechanism by which gap junction (connexin) conductance is modulated is via phosphorylation by protein kinases (Lampe and Lau, 2000; Warn-Cramer and Lau, 2004; Moreno and Lau, 2007). There is evidence that PKA is involved with the action of dopamine on horizontal cells (Lasater, 1987) and PKG is involved with the action of NO (McMahon and Ponomareva, 1996). However, there have been no studies that investigated the effect of protein kinase inhibitors on the reduced coupling produced by dark-suppression, light-adaptation or flickering light.

1.12 Modulation of fish horizontal cell axon terminal coupling

As described in Section 1.5, gap junction coupling between fish horizontal cells occurs at two level: at the level of the somata (HCS) and at the level of the HATs. All of the studies described above concerning the effect of light condition and neuromodulators collected data from HCS. A key question is whether or not the source of the

neuromodulators would be appropriately positioned to influence HATs? In fish retina,

the processes of dopaminergic plexiform cells in the outer retina are mostly in the region

of HCS, but there are also contacts onto HATs, albeit less dense (Yazulla and Zucker,

1988; Van Haesendonck et al., 1993). D1 dopamine receptors have also been identified

by immunohistochemistry on HATs, again less densely than for HCS (Mora-Ferrer et al.,

1999). There is only one study that has examined the effect of dopamine on HATs and,

despite the evidence of dopaminergic innervation described above, it reported no effect of

either dopamine or bright light exposure on receptive-field size (Shigematsu and

Yamada, 1988). With respect to NO, HATs might be better positioned to be influenced

by NO released from amacrine cells. In addition, there is some indication that HATs

might, like HCS, contain nNOS (Liepe et al., 1994). No studies have examined the effect

of NO specifically on HATs.

1.13 Tracer coupling of horizontal cells

The receptive-field size of horizontal cells can be assessed by recording the light

responses of horizontal cells (usually by intracellular recording) to various types of

stimuli. One method (e.g. Daniels and Baldridge (2011) is to translate a spot or slit of

light across the receptive-field to determine the distance over which responses can be

recorded. A more common approach is to compare the responses to stimuli (small spot)

aligned with the cell being recorded with response to stimuli displaced by the cell (e.g., a

ring of light or annulus). Well-coupled horizontal cells should show strong responses to

both spot and annulus stimuli. When cells are uncoupled, the response to the annulus

decreases (signals produced in cells stimulated by the light of the annulus no longer reach

the recorded cell because of decreased gap junction coupling). When gap junctions are

uncoupled, the input resistance of horizontal cells increases and, therefore, the response

to the stimulus aligned with the recorded cell can increase. However, this requires quite

small stimuli, to limit the contribution of spatial summation that (like the response to the

annulus) is affected by the extent of gap junction coupling. Generating and aligning such

a small stimulus with the recording electrode can be very challenging. Therefore, the

most common approach is to use moderate size spots and large annuli and take the ratio

of the response amplitudes of each as an indicator of receptive-field size (Baldridge and

Ball, 1991; Pottek et al., 1997; Djamgoz et al., 1998; Weiler et al., 1998; Weiler et al.,

2000; Xin and Bloomfield, 2000; Furukawa et al., 2002). Treatments that uncouple

horizontal cells would decrease the responses to both spot and annulus stimuli, but the

decrease of the annulus will be greater, resulting in a decreased annulus/spot ratio.

A limitation is that none of these electrophysiological measures of receptive-field

size necessarily report changes in gap junction coupling. The network of coupled

horizontal cells can be described using cable theory (Lamb, 1976), with the response to

stimuli distant (x) from the cell being recorded (distance 0) approximated by:

$$V_{(x)} = V_{(0)}e^{-\lambda/x}$$

where

$$\lambda = \sqrt{\frac{Rm}{Ri}}$$

and λ is the length constant, Rm is the membrane resistance, and Ri is the internal

resistance. In other words, at one length constant distance, the response of a stimulus will

decrease by 1/e of the response when the stimulus is centred over the cell being recorded.

In this model, gap junction resistance would be part of Ri but it is evident that λ, and

therefore receptive-field size, could decrease either by an increase in Ri (increased gap junction resistance) or decreased membrane resistance (Rm). Therefore, to really know that changes in horizontal cell receptive-field size are due to gap junctions requires a different approach.

This approach, as described above, is to use gap junction permeable dyes or tracers. The most common approach used is to inject dye or tracer using intracellular recording techniques and current injection (iontophoresis) (Schmued and Heimer, 1990; Bloomfield et al., 1995). However, this can be quite challenging because the fine tips of intracellular pipettes do not necessarily easily pass dye or tracer. An alternative to dye or tracer injections was developed by the laboratory of Stuart Mangel (Choi et al., 2012). Their objective was to load photoreceptors with the gap junction-permeable tracer Neurobiotin. This is nearly impossible to do via intracellular iontophoresis because of the very small size of the cells. Instead, they developed "cut loading," where a blade dipped in Neurobiotin was used to make cuts in isolated retina. Photoreceptors along the cut edge were loaded with Neurobiotin and the tracer spread via gap junctions to adjacent cells, away from the cut. They also reported loading of other types of retinal neurons, including horizontal cells, making it possible that this approach could be used, instead of intracellular iontophoresis, to assess horizontal cell gap junction coupling.

1.14 Thesis objectives

The objective of the thesis work presented here was to study further the mechanism of horizontal cell light-adaptation, with a focus on the role of NO. The guiding hypothesis is that NO, via the activity of PKG, is the mechanism by which horizontal cell

gap junctions are uncoupled following exposure to periods of bright, sustained illumination (light-adaptation).

I first (Chapter 2) attempted to use bulk-loading of Neurobiotin, using a modification of cut loading ("stab loading"), to load horizontal cells in the isolated goldfish (*Carassius auratus)* retina that then could be used to assess changes in gap junction coupling. Although this method did not consistently load horizontal cell somata (HCS), it did produce loading of HATs and the spread of Neurobiotin was affected by light conditions and drug treatments that reduce gap junction coupling between HCS. In Chapter 3 I used this method to study the mechanism of the modulation of goldfish (*Carassius auratus*) HAT gap junction coupling with an emphasis on NO and PKG and, for comparison, PKA. Additional experiments examined dark-suppression and the effect of flickering light, assessing the impact of NO, PKG and PKA. Lastly, in Chapter 4, I determined if the PKG inhibitor, shown to have an effect on the reduction of HAT gap junction coupling produced by bright light (light-adaptation), had similar effects on goldfish (*Carassius auratus*) HCS receptive-field size assessed using intracellular recording, and on cut loaded Neurobiotin spread between HCS in mouse (*Mus musculus*) retina. An Appendix is also provided, containing data from a side project suggesting that dopaminergic interplexiform cells in fish retina also express the light-sensitive photopigment melanopsin.
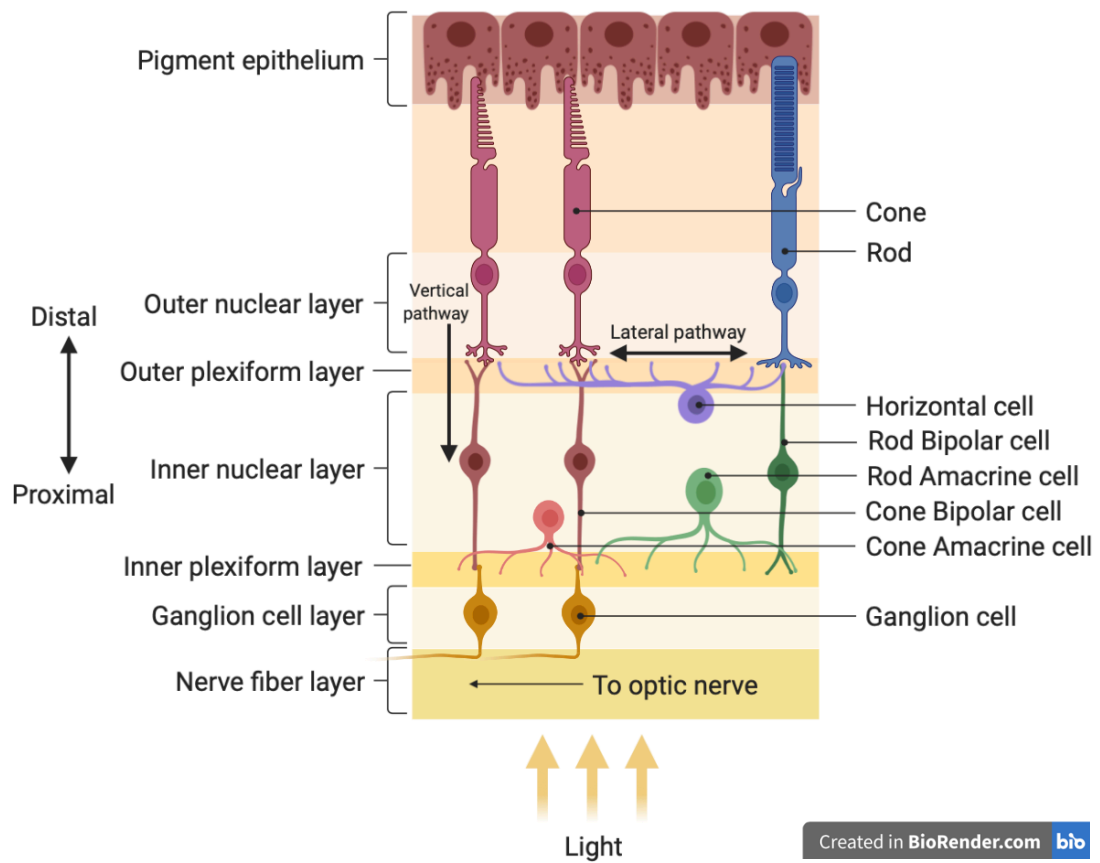
Figure 1.1. Diagram of the vertebrate retina.

Created with BioRender.com
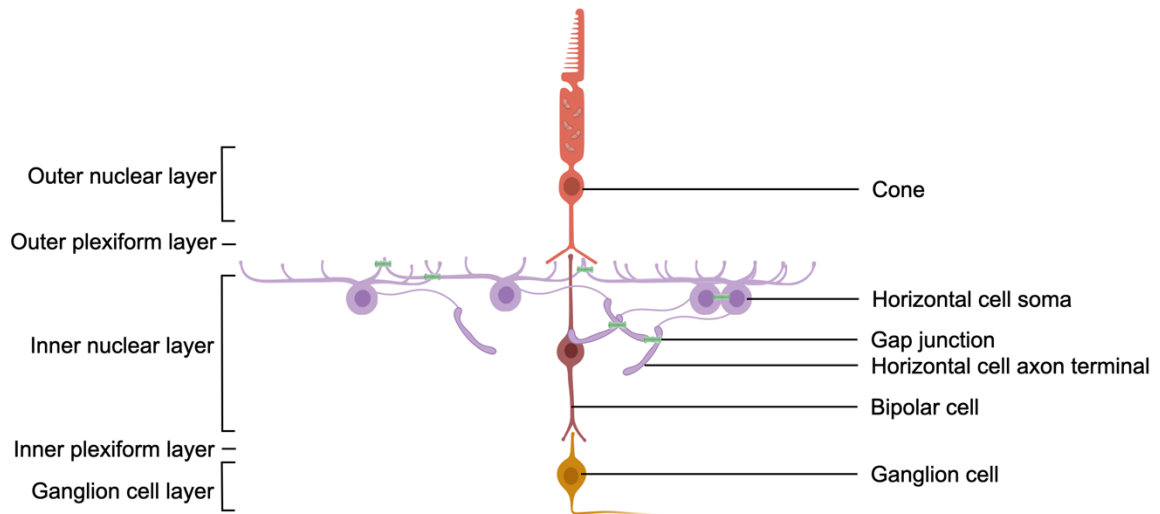
Figure 1.2. Diagram showing the connectivity between cone horizontal cells and neighbouring retinal neurons.

Horizontal cell somata give rise to neurites that are positioned laterally within the cone synaptic terminal while ON bipolar cells dendrites are positioned centrally within the cone terminal. Horizontal cells form gap junctions with each other at their neurites, somata, and HATs. A HAT is also depicted contacting a bipolar cell in the INL.

On-center ganglion cell

Off-center ganglion cell
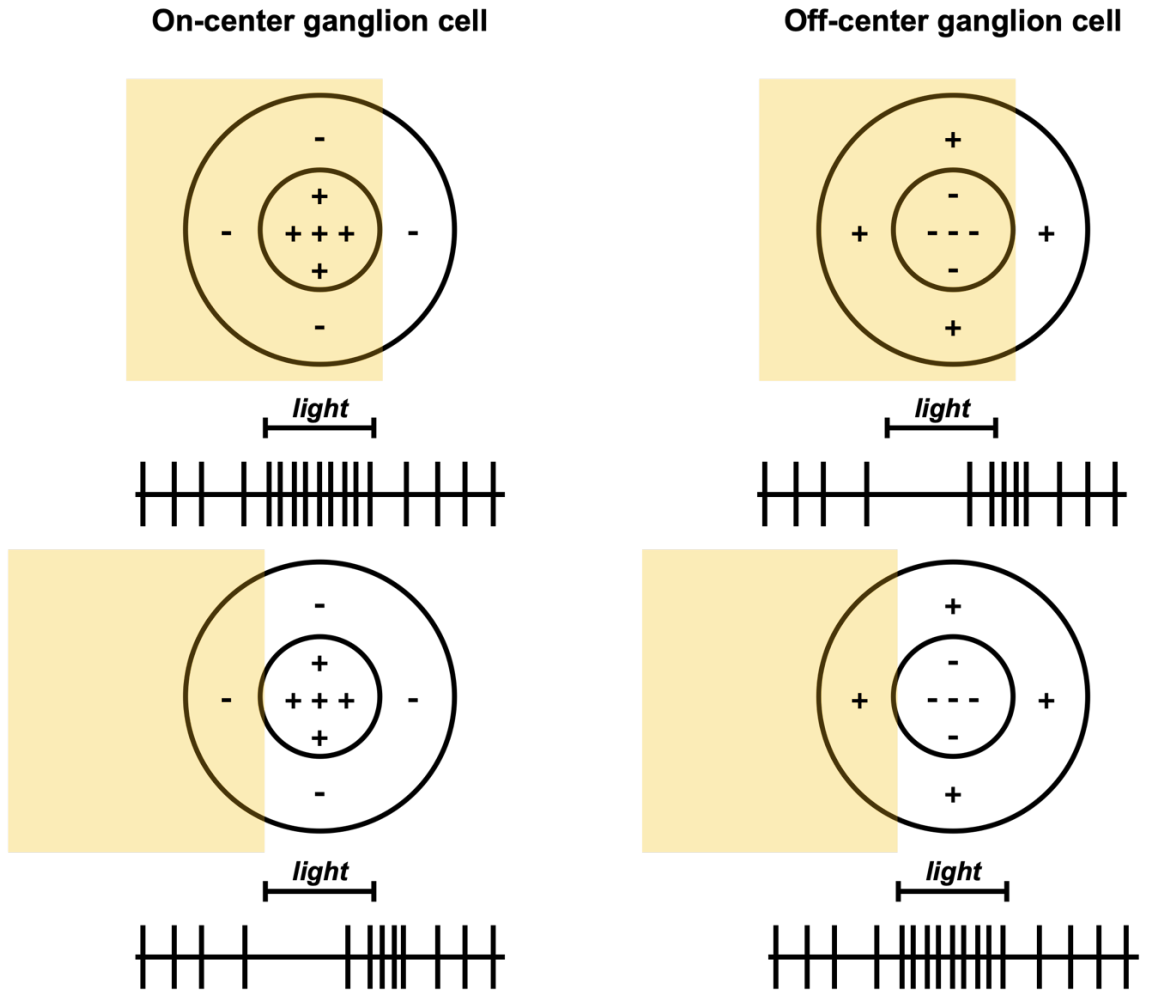
Figure 1.3. Ganglion cell center-surround receptive field responses.

On-center ganglion cells respond with trains of action potentials to light that falls within the center of their receptive field (top left). When an edge of light overlaps with the surround of an on-center ganglion cell, the stimulation of its negative surround results in a dampening of responses (bottom left). The opposite responses are true for off-center ganglion cells (right). Figure adapted from (Kandel et al., 2000).

Light condition	Receptive-field size	Responsiveness
Dark-suppression	↓	↓↓
Light-sensitization	↑	↑↑
Light-adaptation	↓	↓

Table 1.1. Summary of triphasic coupling in horizontal cells.

Dark-suppression is characterized by reduced horizontal receptive field and gap junction coupling and a suppression of horizontal cell responses to light. Light-sensitization results in increased horizontal cell coupling and larger light responses. Light-adaptation results in decreased horizontal coupling and some reduction in responsiveness.

CHAPTER 2:
STAB LOADING HORIZONTAL CELLS IN THE GOLDFISH RETINA WITH NEUROBIOTIN

2.1 Introduction

As described in Section 1.3, gap junctions are transmembrane proteins (connexins) that join the interior of one cell with that of one or more adjacent cells. The resulting pores allow small molecules, ions and electrical current to flow from one cell to another. Although many types of retinal neurons are coupled by gap junctions, a prominent feature of retinal horizontal cells is gap junction coupling (Völgyi et al., 2013). In teleost fish, cone-driven horizontal cells are gap junction coupled at the level of their somata, in the outer part of the INL, and also at horizontal cell axon terminals (HATs) located in the middle part of the INL (see Section 1.5). Gap junction coupling of horizontal cells results in receptive-fields much larger than their dendritic fields and this makes horizontal cells well suited as a source of the surround component of center-surround receptive fields of bipolar and ganglion cells (see Section 1.4).

Horizontal cell gap junction coupling is not static but is modulated by the level of ambient illumination (see Section 1.6) or treatment with exogenous neuromodulators (Section 1.8). The focus of this thesis is to explore connections between light exposure and possible endogenous modulators, in particular nitric oxide, on horizontal cell gap junction coupling. Although electrophysiological recording of horizontal cell light responses can reveal the impact of reduced gap junction coupling, receptive-field size can be altered by changes of membrane resistance, not necessarily changes in gap junction coupling (see Section 1.12). A direct approach to studying gap junctions is dye or tracer coupling where gap-junctional permeable molecules, that can later be visualized by microscopy, are introduced into the interior of a cell with the movement of the dye or tracer from one cell to another reflecting the extent of gap junction coupling.

2.1.1 Tracer injection as a method to visualize coupled neuronal networks

The molecule that was first used extensively to study gap junctions was the fluorescent dye Lucifer yellow (MW ~444 g/mol) (Stewart, 1978). Still in use today, Lucifer yellow is typically injected into cells using glass pipettes via current injection (iontophoresis). Lucifer yellow revealed gap junction coupling of horizontal cells in a variety of vertebrate retinas (Kaneko, 1971; Dacheux and Raviola, 1982; Teranishi et al., 1983; Kaneko and Stuart, 1984; Piccolino et al., 1984) but the spread of the dye was much less than the receptive-field size (Bloomfield et al., 1995) and in many retinal neurons Lucifer yellow was restricted to the injected cell (suggesting that these cells were not coupled by gap junctions).

Neurobiotin was introduced by Vector Labs in the early 1990s as an alternative to biocytin, another neuronal tracer which, though it had a smaller molecular weight than Lucifer yellow (~372 g/mol), had relatively poor water solubility. This made it difficult to introduce levels of biocytin via iontophoresis sufficient for high contrast visualization (Huang et al., 1992). Neurobiotin has a molecular weight of ~286 g/mol and solubility sufficient for iontophoresis. In contrast to Lucifer yellow, the spread of Neurobiotin (iontophoretically injected) is much greater within gap junction coupled networks, including horizontal cells (Vaney, 1991; Dacey and Brace, 1992; Ammermüller et al., 1993; Mills and Massey, 1994). In addition, Neurobiotin revealed evidence of gap junction coupling between populations of retinal neurons that were not previously suspected to possess gap junctions (Vaney, 1991; Hidaka et al., 1993). Although molecular weight is often emphasized as a key criteria for the gap junction permeability of a dye or tracer, the charge of the molecule can also be a factor (Lucifer yellow is negatively charged, Neurobiotin is positively charged) and the size of the molecule in

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solution (related to size but not necessarily linearly) can also be a factor (Kanaporis et al., 2011).

2.1.2 Bulk loading of Neurobiotin

As indicated above, a common approach to load horizontal cells with tracer is via intracellular recording techniques and current injection (iontophoresis). However, this can be quite challenging because tracer does not necessarily pass easily out of the fine-tipped glass pipettes used for intracellular impalement. Under these conditions it can be impossible to know when passing current into the cell through a tracer-filled pipette that any tracer exits the pipette and enters the cell. This means that success rate (a successful injection) can be low. Another disadvantage of using intracellular recording techniques to inject tracer into retinal neurons is the need to use light stimuli to verify cell impalement. For studies of the effect of light on retinal neurons (such as triphasic adaptation of horizontal cells, see Section 1.7) the use of stimuli could alter the very properties of the neurons being studied. This is especially a problem in the case of horizontal cell dark-suppression, where light exposure can readily shift horizontal cells to the light-sensitized condition (Baldridge et al., 1995; Baldridge, 2001).

An alternative to iontophoretic injection of tracer is bulk loading, applied by larger scale physical injection into isolated retina. The laboratory of Stuart Mangel (Choi et al., 2012) developed "cut loading," where a blade dripped in Neurobiotin was used to make cuts in the retina and, by doing so, loaded retinal neurons. Although their focus was on tracer coupling between goldfish photoreceptors, they also suggested that other types of retinal neurons, including horizontal cells, might be loaded by this technique making it possible that this approach could be used, instead of intracellular iontophoresis,

to assess horizontal cell gap junction coupling. They were able to demonstrate examples of cut loading of rabbit horizontal cells, but did not explore this issue further.

I used a modified approach, Neurobiotin "stab loading," for studying horizontal cell gap junction coupling in isolated goldfish retina. This method did not consistently load HCS with Neurobiotin but did label HATs (see Section 1.5) revealing spread distant from the stab under the illumination condition (light-sensitization) known to promote gap junction coupling between horizontal cell somata (HCS) (Section 1.6). Manipulations that are known to reduce HCS gap junction coupling, including different lighting conditions (see Section 1.6) or drug treatments (see Section 1.8) reduced greatly the spread of Neurobiotin. These observations suggest that stab loading Neurobiotin can be used to study tracer spread through HAT gap junctions.

2.2 Methods

2.2.1 Light conditions and experimental protocol

All experiments were conducted with approval from the Dalhousie University Committee on Laboratory Animals. Goldfish (approximately 10-12 cm in length) were obtained from the Dalhousie University Aquatron and housed in an aquarium and maintained under a 12-hour light/ 12-hour dark cycle.

Experiments were done 4-5 hrs into the daily light cycle and always began with goldfish being placed in the dark for approximately 1 hr. This allowed the retinas to be more easily isolated from the globe as brief darkness results in retraction of rods, decreasing the adherence of the retina to the retinal pigmented epithelium (RPE) (Burnside and Nagle, 1983a). Under dim red light (~4 lux) the fish were decapitated and pithed and the eyes enucleated. Using a scalpel blade and scissors, the anterior portion of

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the eye (containing the cornea and lens) was removed, producing an eyecup containing the posterior layers of the eye: retina, RPE, choroid and sclera. The eyecup was then inverted onto filter paper and the sclera gently lifted using fine forceps. The retina remained adherent to the filter paper and the choroid and RPE were lifted with the sclera. With fine scissors inserted in the space between the RPE and the retina, the optic nerve was cut, allowing the sclera, choroid and RPE to be removed completely with the retina isolated and adherent to the filter paper. The isolated retinas were then maintained in carbogen (95% O_2, 5% CO_2) bubbled Ringer's solution (pH 7.4; Table 2.1).

After dissection, retinas were maintained in Ringer's solution under dim red light for 10 min. Retinas were then subject to one of the following four lighting conditions: 1) maintained in complete darkness for 1 hr (dark-suppression); 2) maintained for an additional 15 min under dim red light (light-sensitization); 3) exposed to fluorescent room lighting (~200 lux) for 15 min (light-adaptation); 4) exposed to flickering light (2 Hz, 250 msec, ~150 lux) produced by a white light-emitting diode strobe light (Enuoli, Bay Shore, NY) for 15 min. At the end of each time point Neurobiotin was applied (see Section 2.2.2) and then retinas were maintained for an additional 30 min under the same lighting condition (darkness, dim red light, room light, flickering light) and then placed in fixative (see below) for 10 min under the same lighting conditions. For experiments involving prolonged darkness (dark-suppression) retinas were manipulated using an infrared imaging system (Find-R-Scope, FJW Optical Systems, Palatine, IL).

In some experiments drugs (see Table 2.2) were added to the Ringer's solution as soon as retinas became subject to one of the experimental light conditions as described above and remained present for the remainder of the experiment. In most cases this

means that drug treatment occurred over the 45 min, during the 15 min prior to stab application of Neurobiotin and the 30 min period following the stab. As dark-suppression requires a long period of darkness, in these case drug exposure was 90 min, during the 60 min of darkness before the stab and the 30 min following the stab. Drugs used included dopamine (DA), DETA NONOate, all-*trans*-retinoic acid (RA), carbenoxolone (CBX), and meclofenamic acid (MFA). The drug concentrations used were based on previous reports of concentrations that were effective, where possible in studies of teleost retina (see Table 2.2). Stock solutions were made by dissolving in water or dimethyl sulfoxide (DMSO) (Table 2.2) and then dissolved in Ringer's solution at the desired concentration.

2.2.2 Neurobiotin application and histochemistry

Neurobiotin (Burlingame, CA) was applied to isolated retinas by ejecting a small amount of 5% Neurobiotin (approximately 0.5 µL, dissolved in water) at the tip of a 1 µL Hamilton syringe needle and then plunging the needle into the retina. Each retina received a "stab" ~4 mm from the edge of the retina and positioned as centrally as possible (near the optic nerve). After 30 min (under the same lighting condition) retinas were fixed using 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 10 min. Retinas were then washed 3 times for 7 min with 0.1M phosphate buffer.

To fluorescently tag Neurobiotin for microscopic imaging, retinas were incubated overnight at 4°C (refrigerator) in Streptavidin conjugated to Alexa Fluor 488 (Invitrogen ThermoFisher, Ottawa, ON; #S32354), 1:150 in 0.1 M phosphate buffer. After additional washes (3 times, 7 min each) retinas were mounted on glass slides, cover-slipped using 50% glycerol in 0.1M phosphate buffer media.

2.2.5 Image acquisition and processing

Neurobiotin/Alexa 488 labelling in flat-mounted retinas was imaged using a Nikon Eclipse C1 confocal microscope employing a 20X (0.75 N.A.) Plan Fluor objective. To visualize Alexa 488 fluorescence by confocal microscopy, a 488 nm laser was used for excitation with emission passed through a 520 ± 20 nm filter. The INL was localized in relation to the GCL, IPL and OPL by altering the z-depth (focus) with the aim of identifying HCS and HATs loaded with Neurobiotin. An image was first captured at the location of the stab. If the spread of Neurobiotin beyond this field of view was apparent, additional images were captured along the extent of the spread and including the most distant region, to the point where Neurobiotin labelling was not seen to spread further. When Neurobiotin spread beyond the field of view of the image with the stab, the multiple images were stitched and measurements of Neurobiotin spread determined using Fiji by Image J (Schindelin et al., 2012). Neurobiotin spread was measured as the distance in μm from the edge of the stab to the farthest point where Neurobiotin fluorescence could be visualized by confocal microscopy.

2.2.6 Statistical analysis

All data are presented as mean ± standard deviation ($\bar{x} \pm \mathrm{SD}$). A one-way analysis of variance (ANOVA) and Bonferroni posthoc test corrections for multiple comparisons were run a single time (Prism, GraphPad, San Diego, CA), comparing Neurobiotin spread from relevant groups in this chapter and the following chapter. The control results from this chapter served as the baseline data for further drug experiments throughout this and the following chapter.

2.3 Results

2.3.1 Stab loading of horizontal cell axon terminals in the goldfish retina

Initial attempts to use "cut loading" (Choi et al., 2012) to load goldfish horizontal

cells with Neurobiotin did not produce consistent results and the labelling achieved was

very weak. Motivated by a method developed to load calcium-indicator dye into inner

retinal neurons by "stab loading" (Baldridge, 1996), I applied this approach to the

application of Neurobiotin in the goldfish retina. Initial experiments were done in retinas

subject to dim illumination (light-sensitized) to determine if retinas stab loaded with

Neurobiotin showed evidence of horizontal cell tracer coupling under conditions where

HCS have been shown to have increased gap junction coupling (see Section 1.6). I did

not find I could consistently identify HCS in Neurobiotin stab loaded retinas, but there

was evidence of HCS labelling in some cases (see below). However, the labelling of a

network of processes within the INL was consistently evident in light-sensitized retinas

(Fig. 2.1 LS). Figure 2.1 LS shows confocal images of a retina subject to dim light (light-

sensitization) and stab loaded with Neurobiotin. The asterisk indicates the location of the

stab (left panel) and the two adjacent panels show the view distant from the stab, the

middle panel centred 692 µm from the stab, the right panel showing the extent of

Neurobiotin spread that ended at 2121 µm. In most cases the stab produced a space

within the retina where there was no tissue and hence no labelling. Although there was

some degree of Neurobiotin spread in all directions away from the stab, the distance of

spread was not the same in all directions. Therefore, Neurobiotin spread was quantified

by measuring the distance from the edge of the stab in the direction of the greatest spread,

ending when Neurobiotin/Alexa 488 fluourescence could no longer be detected by confocal microscopy.

The network of processes labelled with Neurobiotin after stab loading is characteristic of HATs, structures in the teleost retina that form in the INL at the end of the long (~200 μm) narrow (0.5 μm) axons that arise from all three types of cone-driven HCS (Stell, 1975; Peng and Lam, 1991). HATs consist of long (~200 μm) terminals, with a diameter of 5-7 μm, but tapering at both ends (Stell, 1975). The network of HATs in the INL is formed by HATs from all three types of cone horizontal cells, arranged in random orientations, forming wavy, intertwining processes that often give the appearance of forming loops (see also Fig. 2.5) (Peng and Lam, 1991). Therefore, the criteria used to identify HATs used here, and for the subsequent studies of Neurobiotin stab loaded retina in this thesis, was to search for the presence of processes in the INL with one or more of the features (large, wavy, looping, or intertwining processes) characteristic of HATs. An example can be seen in the bottom-right panel of Figure 2.5, a micrograph depicting HATs loaded by Neurobiotin through the stab method. Presumptive HCS, that were seen distal to the HATs, were occasionally labelled by the stab method feature (see bottom-left panel of Figure 2.5) but such labelling was not a consistent feature of stab loading. The labelling of HATs was most evident at locations distant from the stab (red arrows, middle and right panel of Fig. 2.1 LS) but near the stab HATs were less obvious (red arrow, left panel of Fig. 2.1 LS).

2.3.2. Triphasic adaptation and effect of flickering light

In light-sensitized retinas stab loaded with Neurobiotin, the tracer spread ranged from 1644 μm - 3900 μm from the stab site but on average was 2245 ± 622 μm (n=13)

(Fig. 2.1, LS; Fig. 2.2). In contrast to the tracer spread in light-sensitized retinas, in retinas subjected to darkness (dark-suppression), bright light (light-adaptation), or flickering light, there was less spread of Neurobiotin from the stab site (DS 269 μm, LA 418 μm, FL 211 μm; Fig. 2.1, DS, LA, LS). Within the region of Neurobiotin spread there were indications of presumptive HAT loading (red arrows) but not the extensive intertwinning processes observed in light-sensitized retinas that extended well beyond the region of the stab.

Mean values of tracer spread were compared for statistical signfiicance using ANOVA and Bonferroni multiple comparison. As data in both Chapter 2 and 3 were ultimately compared, the ANOVA was run on all the data in both chapters. The ANOVA provided an F value of 18.97 and p<0.0001. Mean tracer spread was less (p<0.0001) in retinas that were dark-suppressed (269 ± 113 μm, n=13), light-adapted retinas (419 ± 208 μm, n=17) or exposed to flickering light (222 ± 148 μm, n=9) compared to light-sensitized retinas (Fig. 2.2).

There were instances where Neurobiotin labelled other structures, not characteristic of HATs. This labelling was not seen in all cases but when present generally consisted of round to irregularly shaped profiles ~10 μm wide (Fig. 2.1 LA, FL). This labelling is likely due to HCS as adjustment of z-depth (focus) indicated it was just distal to the level where HATs could be visualized. As this labelling was inconsistent, it was not analyzed further.

2.3.3 Effect of neuromodulators

The effects of three neuromodulators known to reduce HCS gap junction coupling (dopamine, all-*trans*-retinoic acid, and NO) on stab loaded Neurobiotin spread were

tested on light-sensitized retinas. Application (at the beginning and throughout the period of the experimental light condition) of 20 µM dopamine, 100 µM all-*trans*-retinoic acid or 100 µM DETA NONOate all reduced the spread of Neurobiotin from the stab site (Fig. 2.3 DA, RA, NO). Within the region of Neurobiotin spread were examples of presumptive HAT loading (Fig. 2.3 red arrows) and labelling of other structures that could correspond to HCS (Fig. 2.3 LS RA).

Mean Neurobiotin spread after drug application (dopamine 551 ± 185 µm, n=12; all-trans-retinoic acid 1081 ± 408 µm, n=6; DETA NONOate 1039 ± 866 µm, n=7) was less (p<0.0001) compared to the spread of Neurobiotin in untreated (incubated in Ringer alone) light-sensitized retinas (Fig. 2.4).

2.3.4 Effect of gap junction blockers

The effects of two drugs reported to uncouple gap junctions, CBX and MFA (Pan et al., 2007), both operating via as yet undefined mechanisms (Salameh and Dhein, 2005), on stab loaded Neurobiotin spread in light-sensitized retinas were examined. 100 µM CBX (data not shown) did not significantly affect the mean spread of stab loaded Neurobiotin (Fig. 2.4; 2311 ± 850 µm, n=3) but 200 µM MFA reduced the spread of stab loaded Neurobiotin (Fig. 2.3, MFA). Within the region of Neurobiotin spread there was evidence of presumptive HAT loading (Fig. 2.3 LS MFA, red arrow) and labelling of other structures that could correpond to HCS. Mean Neurobiotin spread (737 ± 291 µm, n=11) was less (p<0.0001) compared to the spread of Neurobiotin in untreated (incubated in Ringer alone) light-sensitized retina (Fig. 2.4).

2.3.5 Control experiments

Several control experiments were run (using light-sensitized retinas) to ensure that the fluorescent signal detected was due to the presence of Neurobiotin. Retinas stab loaded with Neurobiotin, but not subsequently incubated in Streptavidin/Alexa 488, did not produce labelling. Retinas stabbed with vehicle (water), but no Neurobiotin, and then incubated in Streptavidin/Alexa 488, also showed no signal. In addition, Neurobiotin applied to the surface of the retina (but not stabbed) failed to produce labelling. Lastly, 70,000 molecular weight rhodamine dextran, a gap junction impermeable tracer, applied via stab loading did not produce any obvious loading or tracer spread.

MFA was first dissolved in DMSO prior to being added to Ringer's solution (resulting in 0.25% DMSO). To ensure that this solvent did not affect Neurobiotin spread I tested the effect of DMSO alone (0.25%) on Neurobiotin spread in light-sensitized retinas. DMSO did not influence the spread of Neurobiotin in light-sensitized retinas, with mean spread of Neurobiotin not significantly different ($p > 0.05$) from mean Neurobiotin spread in retina in Ringer alone (2427 ± 500 μm, n=6).

2.4 Discussion

A major objective of the work described in this chapter was to determine if bulk-loading of Neurobiotin, via "stab loading," would label horizontal cells in the goldfish retina and provide a means to study the modulation of horizontal cell gap junction coupling. In principle, such a simple method would be preferable to iontophoretic intracellular injection by allowing for enhanced success rate, greater throughput of experiments, and greater control of light exposure, a key dependent variable affecting horizontal cell gap junction coupling. The results presented suggest that Neurobiotin stab

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loading is useful for the study of horizontal cell gap junction coupling, but specifically coupling at the level of the HATs.

As described in Section 1.5, there are two types of horizontal cells in teleost retinas: 1) cone-driven horizontal cells (3 or more subtypes, each making specific connections with 3 or more types of cones), that each give rise to narrow (0.5 μm diameter) axons; 2) rod-driven horizontal cells, usually a single type without an axon (Stell, 1975). In goldfish, there are three types of cone-driven HCS (H1-H3) and one rod-driven HCS (H4). The HCS are confined to a single layer ~10 μm thick in the distal INL. Stratification of the 4 types of HCS is "rudimentary" (Stell and Lightfoot, 1975) with the outermost cells (H1) occupying the outer 5 μm of the 10 μm layer region containing HCS and all the remaining cells (H2-H4) "compressed" into the inner 5 μm. The axons of the cone-driven horizontal cells run from the distal INL into deeper (proximal) regions of the INL ending as enlarged (5-7 μm diameter) axon terminals (Stell, 1975). HATs are arranged in random orientations and form a characteristic intertwining network throughout the proximal and middle INL (Fig. 2.5) (Peng and Lam, 1991). This was demonstrated most clearly by Peng and Lam (1991). They produced monoclonal antibodies utilizing mice immunized with dissociated goldfish retinal cells. One of the antibodies (AT101) was found to selectively label HATs (see Fig. 2.5) revealing the extensive "intertwining" network of HATs in the proximal three-fifths of the INL.

The light responses of HATs are indistinguishable from those recorded from the HCS from which they originate and, like HCS, HATs have large receptive fields (Kaneko, 1970; Marmarelis and Naka, 1973; Weiler and Zettler, 1979; Yagi, 1986). The properties of HATs could be explained solely by their connection with HCS, but HATs

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are also known to possess gap junctions (Baldridge et al., 1987; Greb et al., 2017) that can pass Lucifer yellow (Kaneko and Stuart, 1984). Therefore, gap junction coupling between HATs could also contribute to their receptive-field size. In fact, the receptive-field size of HATs appears to be larger than the receptive-field of the corresponding HCS to which they connect via an axon (Kaneko, 1970). Yagi (1986) mapped the receptive field of both HCS and HAT, measuring the response to a slit translated away from a HCS or HAT being recorded. The receptive-field size of HATs was larger than that of HCS and the decline of the reponses with translation distance of HCS consisted of two exponential functions whereas, in HATs, the decline was described by a single exponential function. Interestingly, the exponential function of the HATs was similar to one of the two HCS exponential functions. From this Yagi (1986) concluded that HAT gap junction coupling leads to an expanded receptive-field size and that the signal within HATs is communicated back to HCS via the axon, leading to a receptive field of HCS that reflects both intrinsic HCS gap junction coupling and HAT gap junction coupling.

The labelling achieved in goldfish retina by stab loading with Neurobiotin is consistent with the description of the network of HATs within the INL. Such labelling was extensive in retinas exposed to dim light (light-sensitization) and indicates spread of Neurobiotin several mm from the stab site. Given that individual HATs are ~200 μm in length, such spread of Neurobiotin within the HAT network is consistent with movement of Neurobiotin through HAT gap junctions.

The effect of different light conditions on stab loaded Neurobiotin spread recapitulated previously reported changes of HCS gap junction (see Section 1.6). Retinas maintained in dark for a prolonged period (dark-suppression) or exposed to bright

illumination (light-adapted) or flickering light, showed reduced spread of Neurobiotin compared to retinas exposed to moderate illumination (light-sensitized). Neuromodulators that are known to reduce HCS gap junction coupling (dopamine, all-*trans*-retinoic acid, and NO; see Section 1.8), applied to light-sensitized retinas, also showed reduced spread of stab loaded Neurobiotin. The non-specific gap junction blocker MFA also reduced the spread of stab loaded Neurobiotin in light-sensitized retinas, but another gap junction blocker, CBX, did not. Interestingly, in a study of rabbit retina, Pan et al. (2007) found CBX had only a weak effect (even at 400 µM) on HCS tracer coupling whereas MFA completely abolished tracer coupling. Therefore, taken as a whole, the changes of stab loaded Neurobiotin spread with light condition or drug treatments reported here are consistent with previously reported changes of HCS gap junction coupling.

The reduction of Neurobiotin spread in stab loaded retinas subject to light conditions or drug treatments suggests that these treatments reduce HAT gap junction coupling. Also consistent with this conclusion is that the gap junction blocker MFA reduced tracer spread. However, a limitation of stab loading is that definitive loading of HATs was often less obvious near the stab, especially in cases where Neurobiotin spread was reduced. A possible explanation is that, in some cases, the treatments reduced HAT gap junction permeability such that there would be little Neurobiotin spread even within those HATs near the stab. In fact, in their study of photoreceptor coupling using cut loading, Choi et al. (2012) noted almost no loading of photoreceptors at the edge of the cut under conditions where gap junctions were considered to be the most uncoupled. The effect of different light conditions or drug treatments, relative to the control light-

sensitized conditions, resulted essentially in two degrees of Neurobiotin tracer spread: extended (~2 mm) or reduced (~1 mm or less), with changes consistent with what has been found for the same light conditions or treatments that affect HCS gap junctions. Therefore, I conclude that the spread of Neurobiotin, especially over the extended distance seen in light-sensitized retina, is due to HAT gap junction coupling and that the reduced spread, seen following treatments known to reduce HCS gap junction coupling, is due to a reduction of HAT gap junction coupling.

An issue encountered was that labelling of other elements (not HATs) was apparent in some cases after stab loading. As suggested above (Section 2.3.2), this could be labelling of HCS in the distal INL. Such labelling was not seen consistently and, therefore, was not investigated further.

What other mechanism might explain the spread of Neurobiotin following stab loading? It is possible that the extensive spread of Neurobiotin seen in light-sensitized retina was due to extracellular diffusion with neurons taking up the tracer along the path of diffusion (Huang et al., 1992). I did not find that Neurobiotin added to the retina (without stabbing) produced any labelling but it is not clear that this would necessarily be the same as stab loading as Neurobiotin might not pass easily through the limiting membranes of the retina. However, it seems unlikely that different light conditions, or the range of different types of drug treatments used here, would all (except light-sensitization) reduce diffusion or uptake in isolated retinas giving rise to different degrees of Neurobiotin spread.

The effect of light-adaptation and dopamine reported here, leading to a reduction of the spread of stab loaded Neurobiotin in the goldfish retina, differs from the results of

Shigematsu and Yamada (1988), where neither light-adaptation nor dopamine had an effect on HAT receptive-field size in carp retina. It is difficult to reconcile these results. One issue with the work of Shigematsu and Yamada (1988) is that no group data is provided, with only a single (n=1) example illustrated. It is also surprising that dopamine was without effect given that D1 dopamine receptors have been identified by immunohistochemistry on HATs, albeit less densely than what is seen for HCS (Mora-Ferrer et al., 1999).

Horizontal cells gap junction coupling is thought to be important for the generation of the receptive-field surround of bipolar and ganglion cells, through feedback to photoreceptors (see Section 1.4). Clearly such feedback depends on the neurites of HCS, that are both post-synaptic to photoreceptors and also believed to influence photoreceptor synapses (see Section 1.2). What would be the impact of HATs, and in particular of gap junction coupling of HATs, on the properties of HCS? If it is indeed the case that HAT responses are produced solely by electrical signals received from HCS via the axon (Stell, 1975; Teranishi et al., 1983; Yagi and Kaneko, 1988), it is a reasonable assumption that signals could travel from HATs to HCS. This means that the receptive fields of HATs would complement the receptive-fields of HCS generated by HCS-to-HCS gap junction coupling. How the network of each type of cone-driven horizontal cell would benefit from being coupled at the level of the somata and the axon terminal is not clear. But it would make good sense that the gap junction coupling of HATs would be modulated in a manner similar to that at the level of HCS. If HAT gap junction coupling is not modulated, would this not maintain an enlarged receptive field of HCS, even if HCS were uncoupled?

An alternative function for HATs is through effects on other neurons in the INL. Several electron microscopic studies have indicated synaptic contacts from HATs onto bipolar cells, amacrine cell and interplexiform cells (Marc and Liu, 1984; Sakai and Naka, 1985; Sakai and Naka, 1986; Marshak and Dowling, 1987). The potential significance of these contacts will be explored further in Chapter 5 but, at face value, suggests the possibility of a transfer of surround information to the bipolar cells independent of horizontal cell feedback to photoreceptors (Marshak and Dowling, 1987).

Components	mM
NaCl	130
NaHCO$_3$	20
KCl	2.5
glucose	10
MgCl$_2$	1
CaCl$_2$	0.7

Table 2.1 Ringer's solution composition.

This Ringer's solution was used to maintain goldfish retinas *ex vivo*. The components of the solution were dissolved in type 2 purified H$_2$O. The solution was bubbled with 95% O$_2$/5% CO$_2$ to maintain a pH of 7.4.

Drug	Main working concentration and supporting citation	Source	Solubility (Stock Solution)
Dopamine	20 µM Baldridge and Ball, 1991	Sigma-Aldrich #H8502	H_2O (100 mg/ml)
All-*trans*-retinoic acid	100 µM Pottek and Weiler, 2000	Sigma-Aldrich #R2625	DMSO (40 mg/ml)
DETA NONOate	100 µM Daniels and Baldridge, 2011	Sigma-Aldrich #487957	H_2O (10 mg/ml)
Carbenoxolone	100 µM Pan et al., 2007	Sigma-Aldrich #C4790	H_2O (100 mg/ml)
Meclofenamic acid	200 µM Pan et al., 2007	Sigma-Aldrich #M4531	H_2O (50 mg/ml)

Table 2.2 Drugs used in Chapter 2.

Figure 2.1 Effect of light conditions on Neurobiotin spread in isolated goldfish retina after stab loading.

Confocal photomicrographs showing the effect of dark-suppression (DS), light-sensitization (LS), light-adaptation (LA), and flickering light (FL) on Neurobiotin spread after stab loading (location of stab indicated by large white asterisk). The left panel always shows the region where the stab was made. If Neurobiotin spread extended beyond the region depicted in the left panel, additional panels are added. In the case of two panels the second panel is from an adjacent region, showing where Neurobiotin spread ended. In the case of three panels the spread of Neurobiotin is illustrated by a panel (left) at the stab, a panel (middle) at a retinal location distant from the stab (distance indicated by the value, centered and below the panel) and a third (right) panel where Neurobiotin spread ended. Distance values placed below and to the right of a panel indicates the complete distance of Neurobiotin spread from the edge of the stab. The black arrow indicates the location where Neurobiotin spread was found to end in the image. Red arrows indicate the location of presumptive HATs. Scale bars = 100 μm.

DS

269 µm

LS

...

...

692 µm

2121 µm

LA

...

455 µm

FL

53
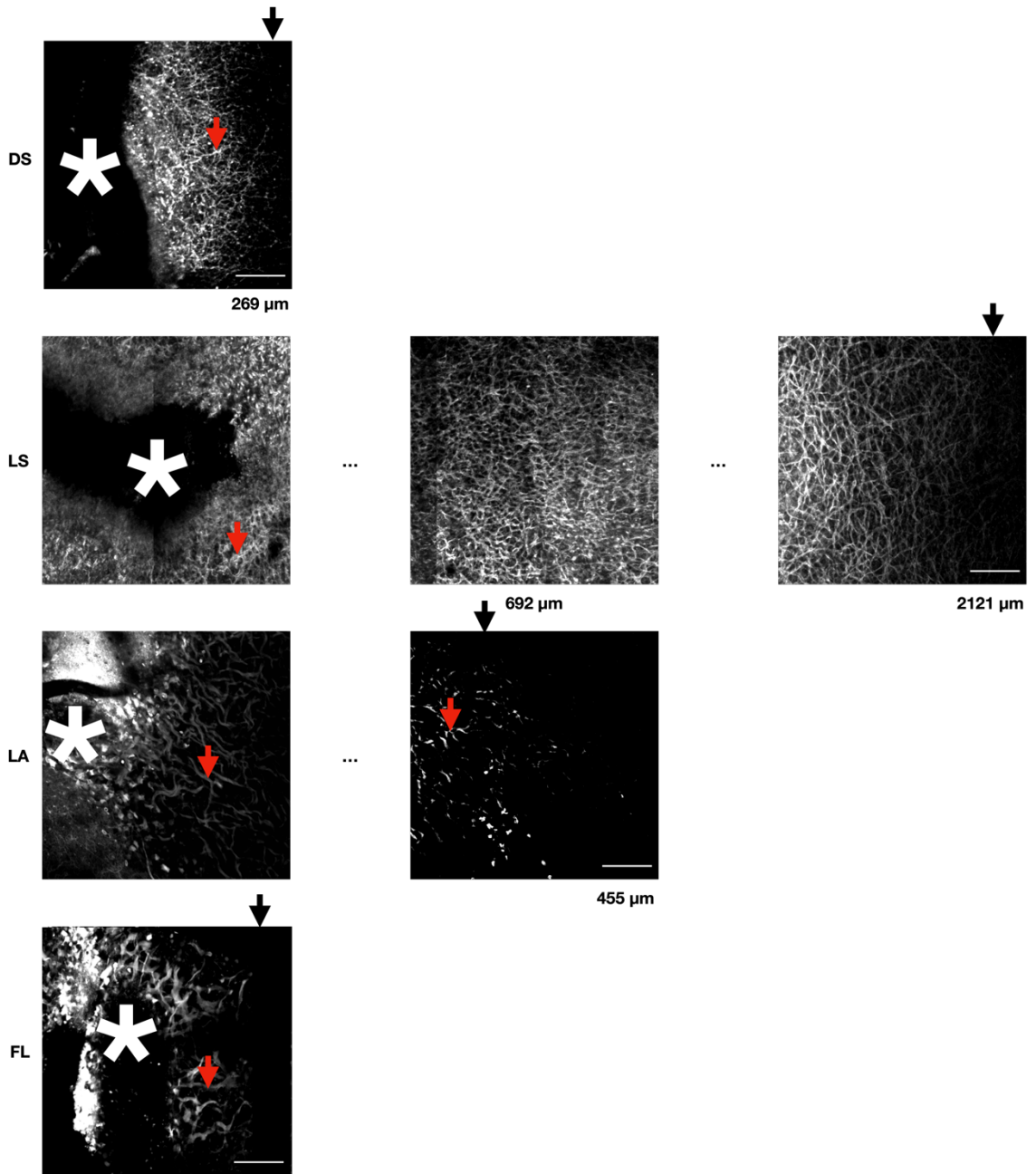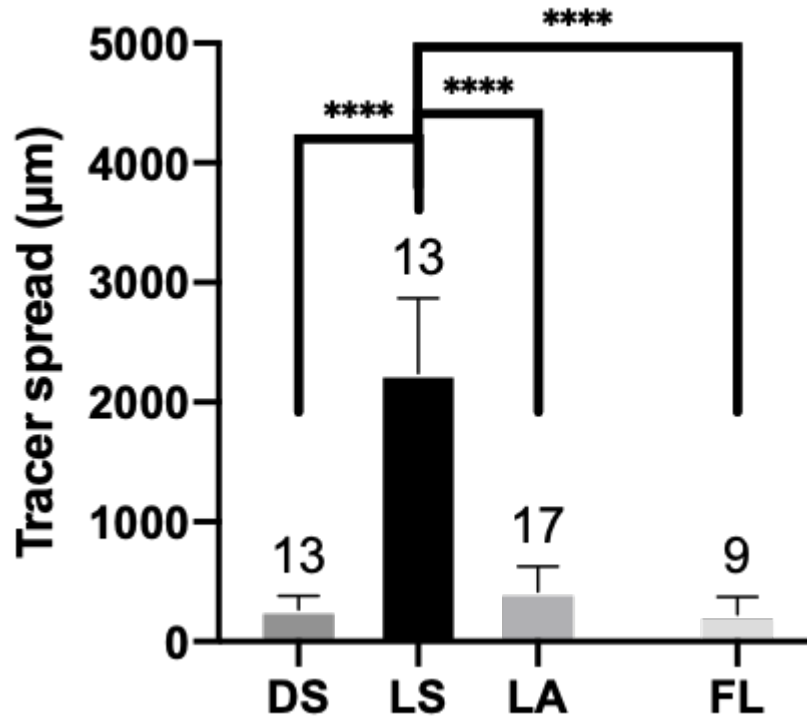
Figure 2.2 Effect of light conditions on mean Neurobiotin spread in isolated goldfish retina after stab loading.

Mean distance ± standard deviation of Neurobiotin spread in μm from the edge of the stab to the farthest detectable labelling under four light conditions: dark-suppression (DS), light-sensitization (LS), light-adaptation (LA), and flickering light (FL). Values provided above each bar indicate sample size. **** indicates $p < 0.0001$
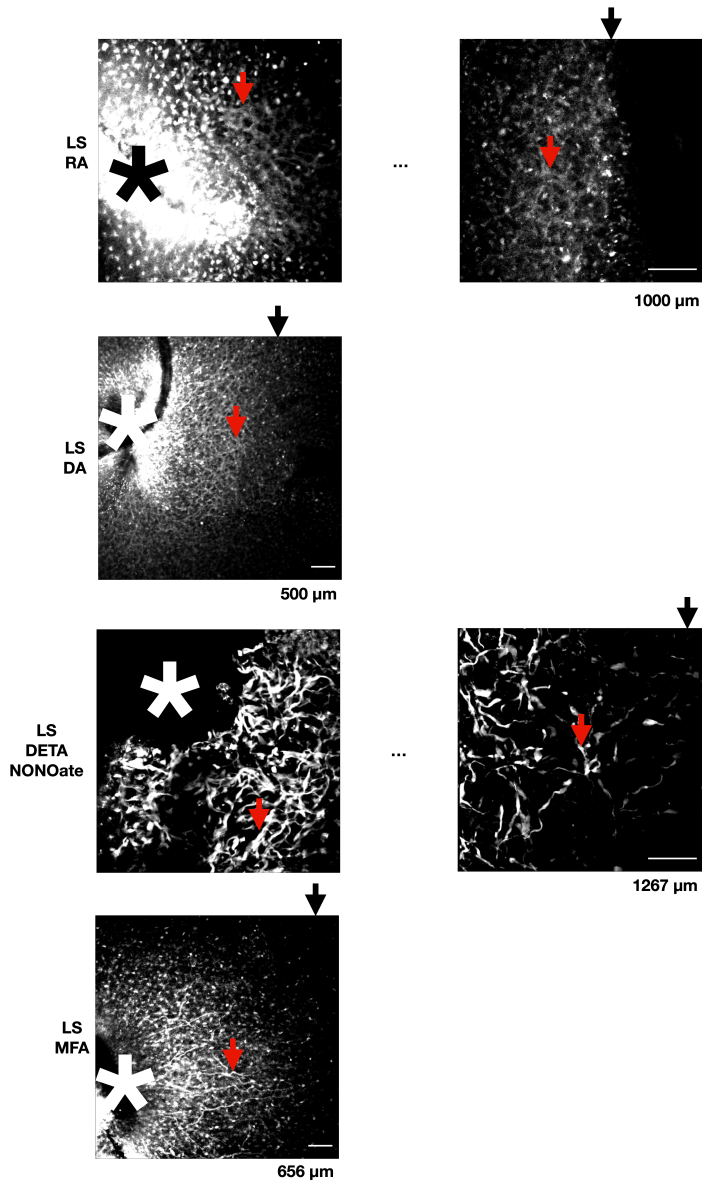
Figure 2.3 Effect of neuromodulators and a gap junction blocker on Neurobiotin spread in isolated goldfish retina after stab loading.

Confocal photomicrographs showing the effect of 100 µM all-*trans*-retinoic acid, 20 µM dopamine, 100 µM DETA NONOate, and 200 µM MFA on Neurobiotin spread after stab loading in light-sensitized retina (location of stab indicated by large white asterisk). The left panels show the region where the stab was made. A second panel, if present, is from an adjacent region, showing where Neurobiotin spread ended. A distance value placed below and to the right of a panel indicates the complete distance of Neurobiotin spread from the edge of the stab. The black arrow indicates the location where Neurobiotin spread was found to end in the image. Red arrows indicate the location of presumptive HATs. Scale bars = 100 µm.
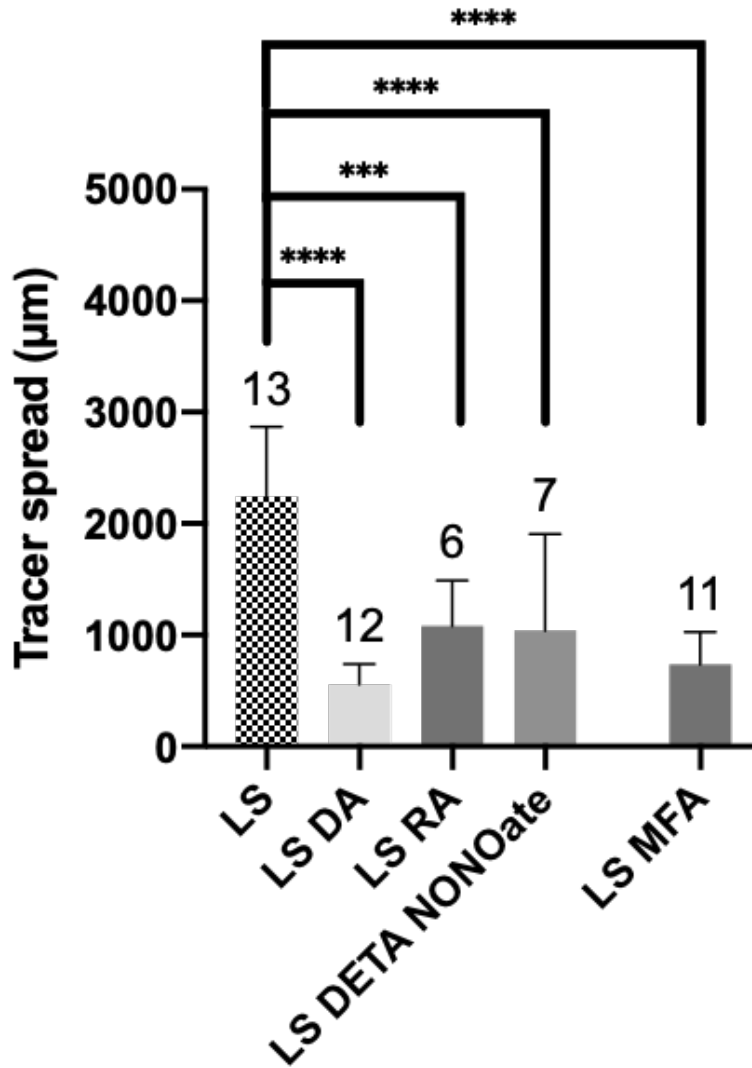
Figure 2.4 Effect of neuromodulators and a gap junction blocker on mean Neurobiotin spread in isolated goldfish retina after stab loading.

Mean distance ± standard deviation of Neurobiotin spread in μm from the edge of the stab to the farthest detectable labelling following four drug treatments applied to light-sensitized retinas: LS DA, 20 μM dopamine; LS RA,100 μM all-*trans*-retinoic acid; LS DETA NONOate, 100 μM DETA NONOate; LS MFA, 200 μM MFA. For comparison, light-sensitized (LS, from Fig. 2.1; checkerboard bar) is included. Values provided above each bar indicate sample size. *** indicates $p < 0.001$, **** indicates $p < 0.0001$.
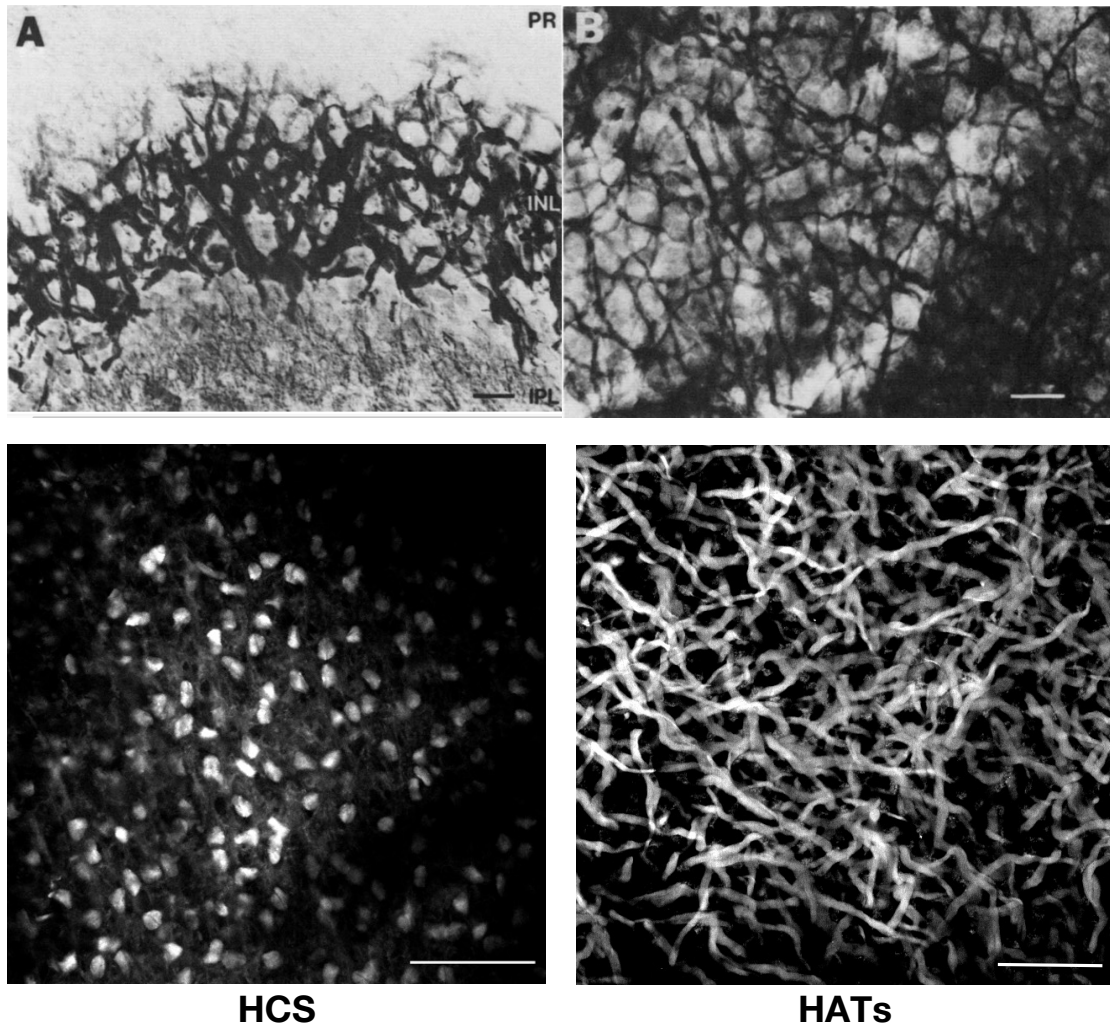
Figure 2.5. Goldfish HAT labelling by the AT101 monoclonal antibody and Neurobiotin-loaded goldfish horizontal cells.

Immunoperoxidase labelling of HAT network in the goldfish retina using the AT101 monoclonal antibody. A) Oblique section of central goldfish retina showing labelled HATs located mostly within the proximal three-fifths of the INL but with occasional projections directed toward the distal INL. B) Wholemount view of at the level of the INL revealing the network of HATs labelled by AT101. Top From Peng and Lam (1991) and used with permission of the publisher. Scale bar = 20 µm.

For comparison, images are provided on the bottom by T. Yousef showing Neurobiotin labelled presumptive HCS (bottom left) and HATs (bottom right). Scale bar = 100 µm.

CHAPTER 3:
MOLECULAR MECHANISMS OF HORIZONTAL CELL AXON TERMINAL ADAPTATION

3.1 Introduction

Horizontal cells are neurons in the INL of the vertebrate retina that receive synaptic input from photoreceptors and, in turn, provide negative feedback to photoreceptor synaptic terminals (see Section 1.2). In teleost retina horizontal cells consist of horizontal cell somata (HCS), typically several types, that form connections with cones and one type associated with rods. Cone-driven HCS possess axons that terminate as horizontal cell axon terminals (HATs) in the middle of the INL (Section 1.5). A prominent feature of horizontal cells is gap junction coupling both at the level of HCS and HATs. First characterized by electron microscopy, more recently specific types of gap junction connexins have been identified connecting horizontal cells. For example, in the carp, which belong to the same family of fish as goldfish (*Cyprinidae*), two connexins (Cx53.8 and Cx55.5) have been shown to be expressed by both HCS and HATs (Section 1.4). Gap junctions endow horizontal cells with a large receptive-field size (Section 1.4) making them well-suited as a source of the receptive-field surround of bipolar and ganglion cells (Section 1.2).

The receptive-field size of HCS is dynamic, influenced by differing levels of ambient illumination (Section 1.6). This is due, at least in part (Section 1.12), to changes of gap junction coupling. The receptive-field size and gap junction coupling of HCS shows three different phases subject to different light conditions (Section 1.6). In retinas maintained in dark for a prolonged (≥ 1 hr) period, both receptive-field size and gap junction coupling are reduced. Because the responsiveness of HCS is also decreased under this condition, this phase has been termed "dark-suppression." Retinas exposed to steady moderate illumination show increased receptive-field size, gap junction coupling

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and responsiveness ("light-sensitization"). Retinas exposed to steady bright light show reduced receptive-field size and gap junction coupling, with modest reductions in responsiveness. In addition, flickering light (applied to light-sensitized retina) can also reduce receptive-field size and gap junction coupling, but by a mechanism thought to be different from steady bright light (light-adaptation) (see below and Section 1.10).

Three neuromodulators (dopamine, NO and retinoic acid) have been identified that reduce HCS receptive-field size and gap junction coupling (Section 1.8). The current state of knowledge suggests that dopamine, acting via D1 dopamine receptors, is involved in the effects of dark-suppression and flickering light on HCS receptive-field size and/or gap junction coupling. D1 dopamine receptors are G-protein coupled receptors that stimulate adenylyl cyclase, and increase cyclic AMP that then activates protein kinase A (PKA) (Section 1.8.1 and Fig. 3.1). The action of dopamine on HCS receptive-field and gap junction coupling is blocked by D1 dopamine receptor antagonists (e.g. SCH-23390); the effect of dopamine is mimicked by application of an adenylyl cyclase stimulator forskolin, by membrane-permeable analogues of cyclic AMP, and intracellular injection of the catalytic subunit of PKA (Section 1.8.1).

Currently it is proposed that NO contributes to the effect of light-adaptation. The common effect of NO is through stimulation of soluble guanylyl cyclase, elevation of cyclic GMP that activates protein kinase G (PKG) (Section 1.8.3, Figure 3.1). The action of NO is mimicked by application of membrane-permeable analogues of cyclic GMP and blocked by intracellular injection of PKG inhibitor (Section 1.8.3).

The potential role of retinoic acid is unclear. Retinoic acid reduces HCS receptive-field size and gap junction coupling and would presumably be produced only in the light

(Section 1.8.2), but there are no studies assessing the effect of endogenous retinoic acid during light-adaptation or flickering light. This is due to the proposed effect of retinoic acid on external retinoic acid receptors either directly on, or intimately associated with, gap junctions, independent of second messengers (Section 1.8.2.), and that no drugs have been identified that block the effect of retinoic acid on HCS receptive-field size or gap junction coupling.

All of the studies described above focused on HCS. Only one previous study (Shigematsu and Yamada, 1988) examined HATs and reported that neither light-adaptation nor dopamine affected HAT receptive-field size. In Chapter 2 I described a method to "stab load" HATs in the isolated goldfish retina with the gap junction-permeable tracer Neurobiotin. I then demonstrated that light conditions (dark-suppression, light-adaptation, flickering light) or drug treatments (dopamine, NO donor, retinoic acid) known to reduce HCS receptive-field size or uncouple HCS gap junctions, reduced the spread of Neurobiotin within HATs suggesting that these same light conditions or treatments also modulate gap junction coupling between HATs.

In this chapter I extend the initial study of HAT gap junction coupling using Neurobiotin stab loading described in Chapter 2. The primary objective was to investigate the mechanism of light-adaptation on HAT gap junction coupling and to test the hypothesis that NO and the cyclic GMP/PKG signalling pathway are involved. Additional studies examined potential mechanisms mediating the reduction of HAT gap junction coupling produced by prolonged darkness (dark-suppression) or flickering light that are thought to involve dopamine and the cyclic AMP/PKA pathway.

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3.2 Methods

3.2.1 Experimental protocol and statistics

Experiments were carried out and analyzed in the same way as described in Chapter 2. All drugs used for the experiments in Chapter 3 (and Chapter 4) are detailed in Table 3.2.

3.3 Results

The results presented below describe experiments that investigated the effect of drugs acting on the nitric oxide/PKG pathway and the cyclic AMP/PKA pathway on stab loaded Neurobiotin spread in isolated goldfish retina. For purposes of comparison, included is mean data from control experiments presented in Chapter 2, specifically mean Neurobiotin spread in control (incubated in Ringer alone) dark-suppressed, light-sensitized, and light-adapted retinas, and also control retinas exposed to flickering light (Fig. 2.1).

3.3.1 Effect of drugs acting on the nitric oxide pathway

3.3.1.1 L-NAME

The role of NO on the modulation of gap junction-coupling of HATs was examined using the NO synthase inhibitor, L-NAME, and stab loaded Neurobiotin tracer coupling. If NO contributes to reduction of gap junction coupling between HATs following light-adaptation, it was expected that L-NAME would interfere with the reduction of Neurobiotin tracer spread produced by light-adaptation. That is, tracer spread would be greater in L-NAME-treated light-adapted retinas compared to light-adapted retinas maintained in Ringer's alone.

Compared to control light-sensitized conditions, 1.0 mM L-NAME did not affect

the spread of Neurobiotin. In the images shown in Fig. 3.2 LS, Neurobiotin spread

reached a distance of 1826 μm withfeatures (thick, wavy, looping processes) associated

with HATs. Mean Neurobiotin spread in L-NAME-treated light-sensitized retinas was

1825 ± 330 μm (n=5; Fig. 3.3 LS L-NAME) which was not significantly ($p>0.05$)

different from mean spread in control light-sensitized retinas (2245 ± 622 μm, n=13; Fig.

3.3 LS). In contrast, L-NAME did affect the reduction of Neurobiotin spread produced

by light-adaptation. Neurobiotin spread in light-adapted retinas in the presence of L-

NAME reached 1534 μm in the example images shown in Fig. 3.2 LA, with labelling

consistent with HATs. Mean tracer spread was 1549 ± 590 μm (n=7) that was not

significantly ($p>0.05$) different from the spread observed in light-sensitized retinas under

control conditions, or L-NAME-treated light-sensitized retina (Fig. 3.3 LA L-NAME

compared to LS or LS L-NAME) but was significantly ($p<0.0001$) different from

Neurobiotin spread in control light-adapted retinas (Fig. 3.3 LA). In contrast, in retinas

exposed to prolonged darkness (dark-suppression) and incubated in L-NAME

Neurobiotin spread was limited to the region near the stab, extending in the example

image shown (Fig. 3.2 DS) 174 μm from the stab site and with evidence of presumptive

HAT labelling. Mean tracer spread was 193 ± 48 μm (n=4) (Fig. 3.3 DS L-NAME) and

was not significantly ($p>0.05$) different from spread achieved under control conditions

(Fig. 3.3 DS). As also described in Chapter 2 (Section 2.3.2), there was in some cases

Neurobiotin labelling of other structures that could correspond to HCS (e.g. Fig. 3.2 LS,

LA).

3.3.1.2 Effect of dibutyryl cyclic GMP

A common mechanism of action of NO is activation of soluble guanylyl cyclase that leads to increased cyclic GMP (see Section 1.8.3). Therefore, if NO is involved with the reduction of HAT tracer coupling during light-adaptation, elevated cyclic GMP should reduce the spread of Neurobiotin in HATs when applied to light-sensitized retinas. To examine this question, light-sensitized retinas were treated with the membrane-permeable analog of cyclic GMP, dibutyryl cyclic GMP (dbcGMP). An example of the effect of 500 µM dbcGMP on Neurobiotin spread after stab is shown in Fig. 3.4A, with labelling consistent with HATs (looping processes) that extended 1114 µm from the edge of the stab. The effect of dbcGMP was examined at 5, 50 and 500 µM (Fig. 3.4B). Compared to control light-sensitized conditions (Fig. 3.4B LS), there was no effect (p>0.05) of 5 µM dbcGMP on mean Neurobiotin spread (2260 ± 966 µm, n=8) but both 50 and 500 µM dbcGMP reduced mean spread (p<0.05 and p<0.0001, respectively) of Neurobiotin when applied to light-sensitized retinas (Fig. 3.4B; 50 µM dbcGMP: 1354 ± 896 µm, n=7; 500 µM dbcGMP: 1009 ± 476 µm, n=6).

3.3.1.3 Effect of KT5823

NO-induced elevations of cyclic GMP can activate PKG that, in turn, could lead to phosphorylation of various target proteins, including connexins (see Section 1.3). To determine if PKG is involved with the reduction of Neurobiotin spread through HAT gap junctions during light-adaptation, the effect of the PKG inhibitor KT5823 was examined. Compared to control, KT5823 did not affect Neurobiotin tracer spread in light-sensitized retina. In light-sensitized retinas treated with 1 µM KT5823 stab loaded Neurobiotin showed spread from the stab site that reached, as illustrated in Fig. 3.5 LS, a distance of

1945 μm with a mean spread of 1780 ± 903 μm (n=7) that was not significantly different

(p>0.05) from mean Neurobiotin spread under control light-sensitized conditions (Fig.

3.6A LS KT5823 vs. LS). KT5823 did affect Neurobiotin spread, compared to control, in

light-adapted retinas. In light-adapted retinas treated with KT5823, as illustrated in Fig.

3.5 LA, Neurobiotin spread reached 1618 μm and mean spread was 3443 ± 950 μm

(n=8) that was significantly (p<0.0001) increased relative to the spread achieved under

control light-adapted retinas (Fig. 3.6A LA KT5823 vs. LA) but not different (p>0.05)

from mean spread in control light-sensitized retinas or light-sensitized retinas treated with

KT5823 (Fig. 3.6A LS, LS KT5823). KT5823 also affected Neurobiotin spread in dark-

suppressed retinas. In retinas treated with KT5823 Neurobiotin spread also extended well

beyond the stab site, reaching 1945 μm in the example shown in Fig. 3.5 DS. Mean

Neurobiotin spread was 1900 ± 969 μm (n=15) which was significantly different from

the spread in control dark-suppressed retinas (Fig. 3.6A DS KT5823 vs. DS). There was,

in some instances, Neurobiotin labelling of other structures (e.g. Fig. 3.5 DS, LS) that

could correspond to HCS (see Section 2.3.2) but this was not a consistent feature.

The choice of 1 μM KT5823 as the concentration used in these experiments was

selected based on testing the effect of a range of concentrations of KT5823 (90 nM – 10

μM) on the effect of light-adaptation on Neurobiotion tracer spread within HATs (Fig.

3.6B). In fact, 1 μM was the only concentration of KT5823 that increased Neurobiotin

spread.

3.3.2 Effects of drugs acting on the cyclic AMP/PKA pathway

3.3.2.1 Effect of dibutyryl cyclic AMP

Another mechanism by which HAT gap junction coupling could be affected is via increased cyclic AMP (see Section 1.8.1). To determine if cyclic AMP can alter gap junction coupling between HATs, light-sensitized retinas were treated with the membrane-permeable analogue dibutyryl cyclic AMP (dbcAMP). As illustrated in Fig. 3.7A, treatment with either 1000 or 10 µM dbcAMP limited Neurobiotin tracer spread to the region near the stab site (126 µm and 282 µm spread, respectively). Although there was some indication of presumptive HAT labelling, these images also show evidence of HCS labelling (see Section 2.3.2). Mean Neurobiotin tracer spread in light-sensitized retina was decreased (p<0.0001) even at 1 µM dbcAMP (mean spread of 391 ± 108 µm, n=5) with similar effects achieved at higher concentrations (at 1000 µM dbcAMP, mean spread was 110 ± 47 µm, n=4; Fig. 3.7B).

3.3.2.2 Effect of H89

The effect of increased cyclic AMP on gap junction coupling is mediated by PKA (see Section 1.8.1). To determine if PKA contributes to the light-dependent changes of HAT gap junction coupling, the effect of the PKA>PKG inhibitor H89 was examined on Neurobiotin spread in light-sensitized retinas subjected to prolonged darkness (dark-suppressed), bright light (light-adaptation) or flickering-light. 0.1 µM H89 did not alter the effect of dark-suppression, with Neurobiotin spread limited to 167 µm in the example shown in Fig. 3.8 DS and mean Neurobiotin spread of 297 ± 115 µm, n= 8) that was not different (p>0.05) from mean spread under control conditions (Fig. 3.9 DS H89 vs. DS). H89 did block the effect of light-adaptation with Neurobiotin spread reaching 2247 µm in

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the example shown in Fig. 3.8 LA and with a mean spread (2424 $\pm$ 745 µm, n=8) that

was significantly greater (p<0.0001) than that under control light-adaptation conditions

(Fig. 3.9 LA H89 vs LA). H89 also blocked the effect of flickering light with

Neurobiotin spread reaching 2198 µm in the example images (Fig. 3.8 FL) and a mean

spread (2080 $\pm$ 542 µm, n=9) that was greater (p<0.0001) than control conditions (Fig.

3.9 FL H89 vs. FL). Additional experiments were done examining the effect of L-NAME

and KT5823 on flickering-light. Neither drug blocked the effect of flickering-light on

Neurobiotin spread (Fig. 3.9, FL L-NAME, FL KT5823; L-NAME: 775 $\pm$ 704 µm,

n=13; p>0.05; KT5823: 302 $\pm$ 303 µm, n=7; p>0.05). As seen in other cases of

Neurobiotin stab loading in this thesis, there were some examples (Fig. 3.8 FL) of

labelling of structures not characteristic of HATs but likely indictive of HCS (see Section

2.3.2). However, as in the other cases, such labelling was not a consistent feature.

3.4 Discussion

In this Chapter I presented studies of the modulation of Neurobiotin spread, used as

a marker of gap junction coupling, between HATs in the isolated goldfish retina

preparation. The primary aim was to explore further the mechanism by which light-

adaptation reduces Neurobiotin spread in HATs (Chapter 2) with the hypothesis that NO

is the mediator of light-adaptation, acting via cyclic GMP and PKG. Additional studies

were done to assess the mechanism that reduces Neurobiotin spread in HATs following

dark-suppression or flickering light (Chapter 2). In this section I will discuss the

interpretation of the results concerning the possible mechanisms mediating light-

adaptation, dark-suppression and flickering light with respect to HAT gap junction

coupling as revealed by Neurobiotin spread. I will then compare and contrast the results

from the studies of HATs with what is known from previous work regarding the modulation of receptive-field size and gap junction coupling of HCS. Additional discussion of the results in this chapter, in context with the studies presented in Chapter 4, will be considered in Chapter 5.

3.4.1 Summary of results

Exposure of retinas, initially maintained under dim light conditions (light-sensitized), to steady bright light (light-adaptation), reduced the spread of Neurobiotin within HATs (Chapter 2). In the presence of the NO synthase inhibitor, L-NAME, the effect of light-adaptation was blocked with Neurobiotin spread not different from that achieved in control light-sensitized retinas. The effect of light-adaptation was also blocked by the PKG inhibitor KT5823 and the PKA>PKG inhibitor H89. In contrast, the reduction of Neurobiotin spread produced by 1 hr of darkness (dark-suppression) was not blocked by L-NAME or H89 but was blocked by KT5823. The reduction of Neurobiotin spread following flickering light was blocked by H89 but not by either KT5823 or L-NAME. These results are summarized in Table 3.1. Lastly, analogues of cyclic GMP and cyclic AMP (dibutyryl cyclic GMP and AMP) reduced Neurobiotin spread when applied to light-sensitized retinas.

3.4.2 Light-adaptation

The primary hypothesis of this thesis is that NO is responsible for the reduction of HAT gap junction coupling by light-adaptation. This was examined using stab loaded Neurobiotin tracer coupling. That the effect of light-adaptation on Neurobiotin spread in HATs was blocked (not significantly different from Neurobiotin spread in light-sensitized retinas) by the NO synthase inhibitor L-NAME (Fig. 3.2 and 3.3) is consistent with a role

for NO as a mediator of light-adaptation. The common pathway for the action of NO is the activation of soluble guanylyl cyclase leading to elevated cyclic GMP and activation of PKG (Garthwaite, 1991). I demonstrated that a membrane-permeable analogue of cyclic GMP, dibutyryl cyclic GMP, reduced the spread of Neurobiotin within HATs (Fig. 3.4) and that the effect of light-adaptation was blocked by the PKG inhibitor KT5823 (Fig. 3.5 and 3.6B). Taken together, these findings are consistent with a pathway for light-adaptation of HATs involving NO, cyclic GMP and PKG.

One issue concerning the use of KT5823 is that I found it was effective only at a single concentration (1 µM; Fig. 3.6B). Not only was it not effective at lower concentrations (90 nM, 0.5 µM) it also was not effective at 10 µM. Studies of various isolated tissue preparations, where an effect of KT5823 has been reported, typically use the drug at 1 µM (Lev-Ram et al., 1997; Michel et al., 2011; Olivares-Gonzalez et al., 2016; Sun et al., 2020) perhaps indicating a lower limit of effectiveness in such preparations and a concentration that is appropriate given the inhibitory constant (K_i) of KT5823 (for PKG, 0.234 µM) (Kase et al., 1987; Hidaka and Kobayashi, 1992). The suggestion of possible KT5823 toxicity (Dostmann and Nickl, 2010) might explain the reduction of Neurobiotin spread at higher concentration.

The PKA>PKG inhibitor H89 also blocked the effect of light-adaptation on Neurobiotin spread within HATs (Fig. 3.8 and 3.9). There are three possible explanations for the effect of H89. First, it is possible that this represents the action of H89 on PKA stimulated by a neuromodulator other than NO. This seems unlikely given the clear effect of L-NAME and in relation to what is known about the effect of light-adaptation on the gap junction coupling of HCS (see Section 3.4.5). Second, the effect of H89 could be

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due to its action on PKG (K_i 0.5 µM) but another (the third) possibility is that there might be an interaction between PKG and PKA as described by Patel et al. (2006). They reported that Neurobiotin coupling between fish (perch) Cx35-expressing HeLa cells was reduced by the NO donor spermine NONOate and that this was blocked partially by KT5823 but was blocked completely by the PKA inhibitor Rp-8-cpt-cAMPs. From these results they proposed a hypothetical model whereby NO-dependent PKG decreases Cx35 permeability (via phosphorylation, either directly or mediated by other effectors) and/or by acting via PKA that that can also phosphorylate Cx35 (Ouyang et al., 2005). A possible mechanism that could explain the cross-talk between PKG and PKA is PKG-mediated phosphorylation of the PKA regulatory subunit RIα that reduces inhibition of PKA leading to activation of PKA without cyclic AMP elevation (Haushalter et al., 2018). Patel et al. (2006) studied Cx35, which mediates gap junction coupling between photoreceptors (Li et al., 2009) but the mechanism could apply to the connexins associated with carp horizontal cells (Greb et al., 2017) given that coupling in this cell type is modulated by both cyclic AMP and cyclic GMP.

3.4.3 Dark-suppression

The reduction of Neurobiotin spread within HATs in isolated retinas maintained in darkness for 1 hr was not blocked by L-NAME (Fig. 3.2 and Fig. 3.3) or H89 (Fig. 3.8 and Fig. 3.9) but was blocked by KT5823 (Fig. 3.5 and Fig. 3.6A). The lack of an effect of L-NAME suggests that NO is not involved in the mechanism by which HAT gap junction coupling is reduced during dark-suppression. That L-NAME did not block the effect of darkness on Neurobiotin spread makes the results obtained using KT5823 unexpected, as it suggests that PKG may be involved in the mechanism even though NO

is not. The lack of an effect of H89 suggests that PKA is not involved and would also suggest that H89 did not affect PKG under these conditions.

A possible explanation for an NO-independent effect of PKG would be the existence of a neuromodulator, other than NO, with effects mediated by cyclic GMP and PKG. One possibility is carbon monoxide (CO) (Verma et al., 1993). There are few studies of CO in the retina. In turtle retina, the CO producing enzyme (heme oxygenase-2) is expressed in photoreceptors, bipolar cells, amacrine cells and ganglion cells and application of exogenous CO increased cyclic GMP immunoreactivity detectable in bipolar and amacrine cells (Cao et al., 2000). However, a mixture of two NO synthase inhibitors, L-NAME and S-methyl-thiocitrulline (SMTC), reduced the effects CO on cyclic GMP immunoreactivity (Cao and Eldred, 2003) suggesting that the effect of CO is, in fact, mediated by NO. Therefore, positing CO as a modulator of dark-suppression does not provide a clear explanation for the effect of KT5823. Other, as yet unidentified, modulators of soluble guanylyl cyclase may exist that could be relevant to the effects of dark-suppression. Consider that before the effect of NO on horizontal cells was discovered it was widely assumed that dopamine was the only neuromodulator of horizontal cell gap junction coupling. Alternatively, an unknown interaction of KT5823 with the mechanism of dark-suppression, independent of PKG, could explain the effect of KT5823 on Neurobiotin spread in dark-suppressed retinas.

3.4.4 Flickering light

The reduction of Neurobiotin spread within HATs produced by flickering light was not blocked by L-NAME or KT5823 but was blocked by H89 (Fig. 3.8 and Fig. 3.9).

These results are consistent with an effect of flickering light on HAT gap junction coupling that does not involve an NO/cyclic GMP pathway but does involve PKA.

3.4.5 Comparison with horizontal cell somata gap junction coupling

All the results presented in this chapter focus on Neurobiotin coupling between HATs. As described in the discussion of Chapter 2, there is little published information about the modulation of HAT gap junction coupling with which to compare the results in this chapter. However, it is possible to consider the results of this chapter with the results of prior studies of the modulation of receptive-field size and gap junction coupling between HCS.

The effect of L-NAME on the reduction of Neurobiotin spread within HATs produced by steady bright light (light-adaptation) is consistent with previous studies of HCS. Daniels and Baldridge (2011) reported that L-NAME reduced the effect of light-adaptation on the receptive-field size of goldfish HCS. However, this study did not verify, using dye or tracer coupling, that the change in receptive-field size involved increased gap junction resistance (see Section 1.12) so it remains to be demonstrated, definitively, that the effect of L-NAME on HCS light-adaptation involves changes in gap junction coupling. Several studies have demonstrated in HCS that treatment with a membrane-permeable analogue of cyclic GMP reduces receptive-field size and/or gap junction coupling (Lu and McMahon, 1997; Pottek et al., 1997; Xin and Bloomfield, 2000), consistent with the effects on HATs reported here. To the best of my knowledge there are no studies examining the effect of PKG or PKA inhibitors on HCS light-adaptation.

The effect of H89 on Neurobiotin spread in HATs following light-adaptation reported here is not consistent with previous studies that showed that HCS light-adaptation was not mediated by dopamine acting at D1 dopamine receptors (Baldridge and Ball, 1991; Umino et al., 1991) and, therefore, presumably not involving PKA. These studies showed that the effect of light-adaptation on HCS receptive-field size and Lucifer yellow coupling was not blocked by treatment with dopamine receptor antagonists (haloperidol or SCH-23390) nor by removing the source of endogenous dopamine using 6-hydroxydopamine. That dopamine is not involved with HCS light-adaptation does not rule out the possible action of a different neuromodulator that could act via PKA, or the possible cross-talk between PKG and PKA described above (see Section 3.4.2). However, both possibilities are speculative and there are no obvious alternative candidates for a PKA-mediated neuromodulator that could affect HCS and HAT gap junction coupling.

No study has examined a possible role for NO or PKG in the reduction of HCS receptive-field size or gap junction coupling after prolonged darkness (dark-suppression). Therefore, it is difficult to evaluate the effect of KT5823 on Neurobiotin spread within HATs in dark-suppressed retinas, as reported here. The leading hypothesis to explain reduced HCS receptive-field size and/or gap junction coupling in dark-suppressed retina is that dopamine, acting via D1 receptors, mediates this effect.

Dark-suppression is defined on the basis of the reduced responsiveness of teleost HCS in isolated retina preparations following prolonged (least 1 hr) of total darkness (Yang et al., 1988a; Baldridge et al., 1995). Suppression did not occur if retinas were incubated in the D1 dopamine receptor antagonist SCH-23390 or if the source of

endogenous dopamine, the interplexiform cells, were abolished by prior treatment with the neurotoxin 6-hydroxydopamine (Yang et al., 1988b). Because prolonged darkness and exogenous dopamine also reduced the spread of Lucifer yellow within coupled HCS, it was concluded that dopamine was responsible for the effects of dark-suppression on HCS gap junction coupling (Tornqvist et al., 1988). Although perhaps it is reasonable to assume that the block of HCS response suppression following darkness by dopamine receptor antagonist or 6-hydoxydopamine also means dopamine is responsible for the reduction of gap junction coupling under the same conditions, this has not been demonstrated definitively. The effect of dopamine on HCS is due to D1 dopamine receptor-mediated increases in cyclic AMP that leads to activation of PKA (Lasater, 1987; McMahon et al., 1989; Dowling, 1991; Roy and Field, 2019). Therefore, KT5823 (on PKG inhibition) of HAT dark-suppression is not consistent with a D1 dopamine receptor-mediated mechanism of dark-suppression in HCS. If dopamine is responsible for the uncoupling of HCS gap junctions during dark-suppression, it would be expected that PKA inhibition should reduce the effect of darkness on HCS gap junction coupling. No such experiment has been undertaken in HCS, but I found that the PKA>PKG inhibitor H89 did not block the reduced spread of Neurobiotin within HATs in dark-suppressed retinas. This result is also not consistent with a role for dopamine in the effect of dark-suppression on HAT gap junction coupling.

The effect of flickering light on HCS coupling is also thought to be mediated by dopamine. Umino et al. (1991) showed that flickering light decreases HCS receptive-field size and gap junction coupling (Lucifer yellow spread) and the effect of flickering light was blocked by the dopamine receptor antagonist haloperidol. The effect of H89

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(and lack of an effect of L-NAME and KT5823) on the reduction of Neurobiotin spread within HATs produced by flickering light, as reported in this chapter, are consistent with a mechanism that involves dopamine acting at D1 dopamine receptors leading to activation of PKA.

In summary, the results of the studies of Neurobiotin spread in teleost HATs reported here are generally consistent with what is known about modulation of HCS gap junctions. In both HCS and HATs, application of exogenous cyclic GMP and cyclic AMP reduces gap junction coupling, and L-NAME blocks the effect of light-adaptation on receptive-field size (in HCS) and Neurobiotin spread (in HATs). Although the effect of protein kinase inhibitors has not been tested on HCS gap junction coupling under differing light conditions, consistent with studies of HCS is the effect of H89, and the lack of effect of KT5823, on Neurobiotin spread in HATs exposed to flickering light, a lighting condition shown previously in HSCs to be mediated by dopamine via D1 dopamine receptors that would increase cyclic AMP and activate PKA. The effect of KT5823 on light-adaptation in HATs was consistent with what is known from studies of HCS, where NO acting via PKG is the likely mechanism. However, the effect of H89 on light-adaptation in HATs was not consistent with results from HCS but could indicate cross-talk between PKG and PKA. Lastly, the effect of KT5823, and lack of effect of H89 on dark-suppression in HATs, is not consistent with what is known about dark-suppression in HCS that is believed to involve a dopamine/PKA pathway.

	Dark-suppressed	Light-adapted	Flickering light
1 mM L-NAME	nd	↑	nd
1 µM KT5823	↑	nd	nd
0.1 µM H89	nd	↑	↑

Table 3.1. Summary of results from Chapter 3

Summary of effect of the effect of NO synthase (L-NAME), PKG (KT5823) and PKA (H89) inhibitors on Neurobiotin spread in HATs under light conditions that normally reduce Neurobiotin spread. nd = no effect; ↑= increased spread in the presence of the drug.

Drug	Main working concentration(s) and supporting citation	Source	Solubility
NO/PKG pathway			
L-NAME	1.0 mM Daniels and Baldridge, 2011	Sigma-Aldrich #483125	H_2O (100 mg/ml)
KT5823	1 µM Based on K_i Dostmann and Nickl, 2010	Sigma-Aldrich #420321	DMSO (1 mg/ml)
dbcGMP	500 µM Pottek et al., 1997	Sigma-Aldrich #370660	H_2O (100 mg/ml)
Dopamine/PKA pathway			
H89	0.1 µM Based on K_i Hidaka and Kobayashi, 1992	Sigma-Aldrich #B1427	H_2O (12 mg/ml)
dbcAMP	1-1000 µM Lasater and Dowling, 1985	Sigma-Aldrich #D0627	H_2O (100 mg/ml)

Table 3.2. Drugs used in Chapter 3 and Chapter 4.

The drugs used in Chapter 3 and Chapter 4 are listed in this table with their working concentrations and supporting citations provided. The companies from where they were purchased, and their product numbers, are also listed.
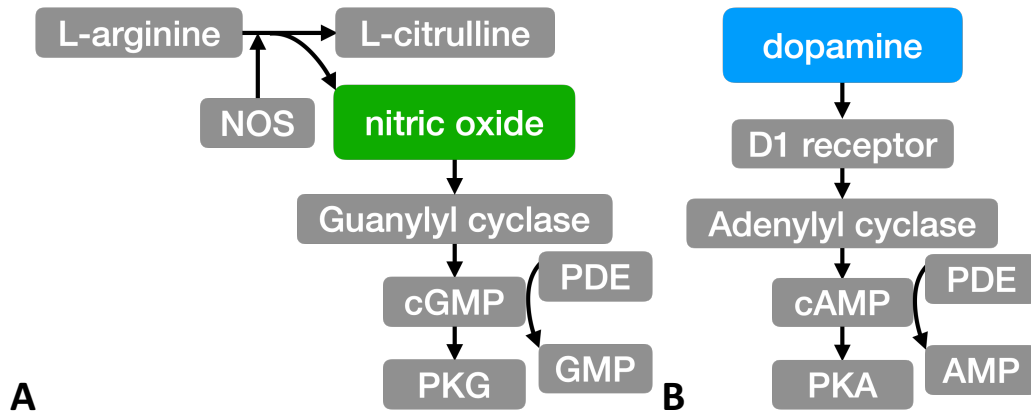
Figure 3.1. NO and DA molecular pathways.

A) The NO molecular cascade that could lead to phosphorylation of gap junction proteins via the action of PKG. NO is a product of the action of nNOS that converts the amino acid L-arginine to L-citrulline. Once NO diffuses into cells it stimulates soluble guanylyl cyclase increasing the production of cyclic GMP that, in turn, can activate PKG. Phosphodiesterases (PDE) can convert cyclic GMP to GMP thereby reducing the activation of PKG. B) Dopamine binds to D1 dopamine receptors increasing adenylyl cyclase activity thereby increasing levels of cyclic that activate PKA that could phosphorylate gap junction proteins. Phosphodiesterases (PDE) can convert cyclic AMP to AMP thereby reducing the activation of PKA.

Figure 3.2. Effect of L-NAME under differing light conditions on Neurobiotin spread in isolated goldfish retina after stab loading.

Confocal photomicrographs showing the effect of 1.0 mM L-NAME on Neurobiotin spread after stab loading in retinas subject to dark-suppression (DS), light-sensitization (LS), and light-adaptation (LA) (location of stab indicated by large white asterisk). The left panel shows the region where the stab was made. If Neurobiotin spread extended beyond the region depicted in the left panel, additional panels are added. In the case of three panels the spread of Neurobiotin is illustrated by a panel (left) at the stab, a panel (middle) at a retinal location distant from the stab (distance indicated by the value, centered and below the panel) and a third (right) panel where Neurobiotin spread ended. Distance values placed below and to the right of a panel indicates the complete distance of Neurobiotin spread from the edge of the stab. The black arrow indicates the location where Neurobiotin spread was found to end in the image. Red arrows indicate the location of presumptive HATs. Scale bars = 100 μm.

Figure 3.3. Effect of L-NAME on mean Neurobiotin spread in isolated goldfish retina after stab loading and under different light conditions.

Mean distance ± standard deviation of Neurobiotin spread in μm from the stab point to the farthest point where labelling was detected. DS=dark-suppression, LS=light-sensitization, LA=light-adaptation, and FL=flickering light. Checkerboard bars indicate control conditions (same data as in Fig. 2.1) and filled bars indicate the same light conditions but in the presence of 1.0 mM L-NAME. Values provided above each bar indicate sample size. **** indicates $p < 0.0001$.

Figure 3.4. Effect of dbcGMP on Neurobiotin spread in isolated light-sensitized goldfish retina after stab loading.

A) Confocal photomicrographs showing the effect of 500 µM dbcGMP on Neurobiotin spread after stab loading (location of stab indicated by large white asterisk). The left panel shows the region where the stab was made. The second panel is from region where Neurobiotin spread was found to end (black arrow) and is also indicated by the value below and to the right of the panel. Red arrows indicate the location of presumptive HATs. Scale bar = 100 µm. B) Mean distance ± standard deviation of Neurobiotin spread in µm from the stab point to the farthest detectable labelling. The checkerboard bar indicates mean value from control LS (same data as in Fig. 2.1) and filled bars indicate LS but in the presence 5, 50 and 500 µM dbcGMP. Values provided above each bar indicate sample size. * indicates $p < 0.05$ and **** indicates $p < 0.0001$.

A
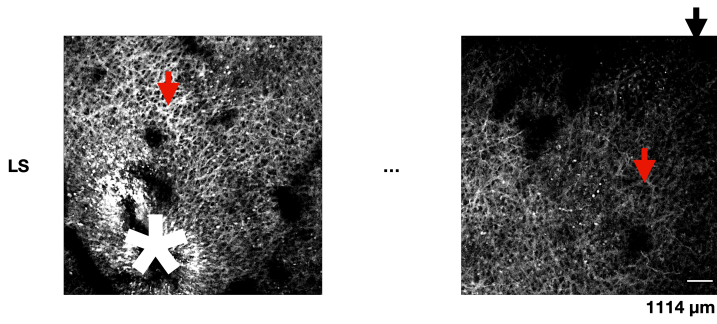
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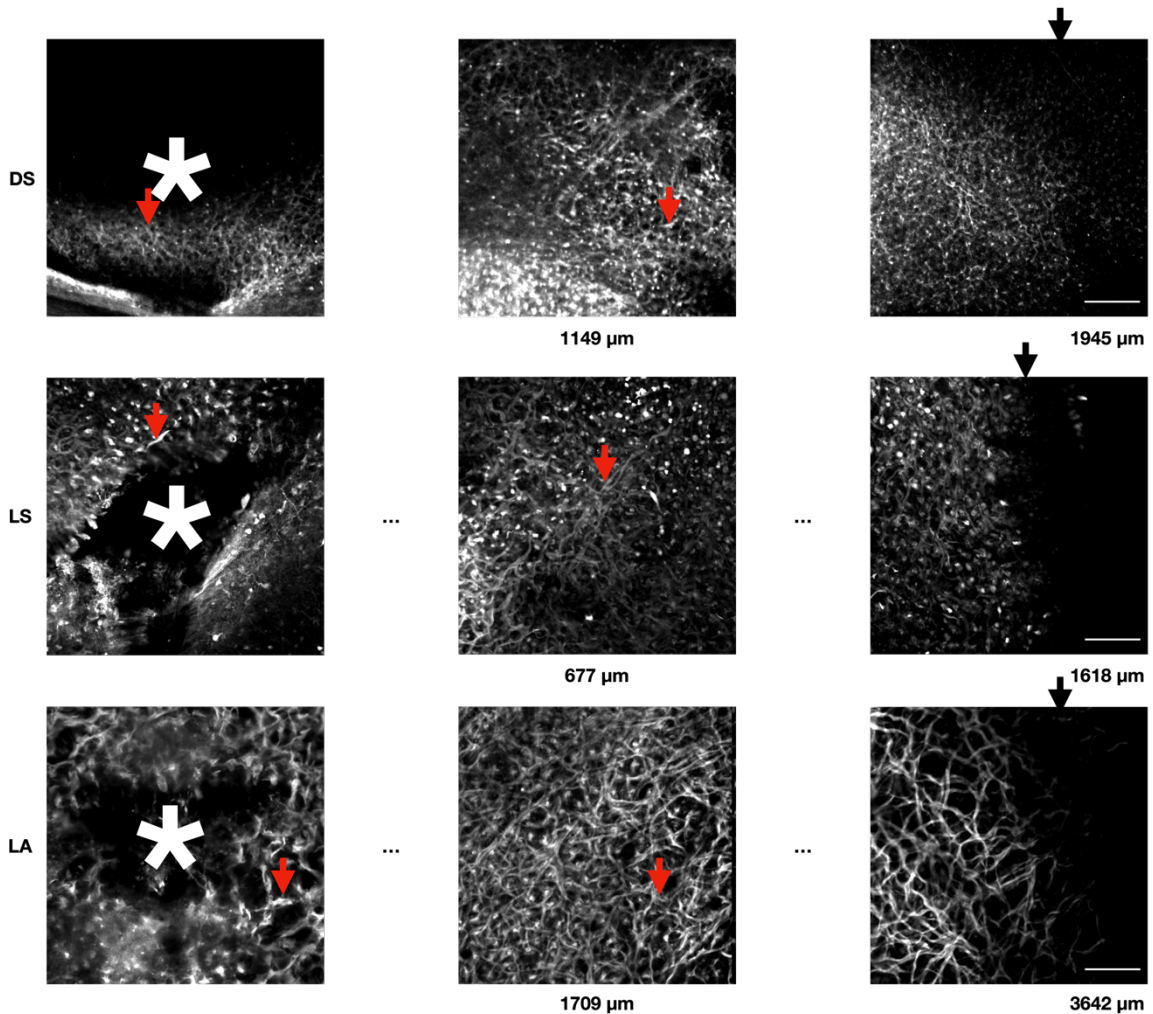
Figure 3.5. Effect of KT5823 under differing light conditions on Neurobiotin spread in isolated goldfish retina after stab loading.
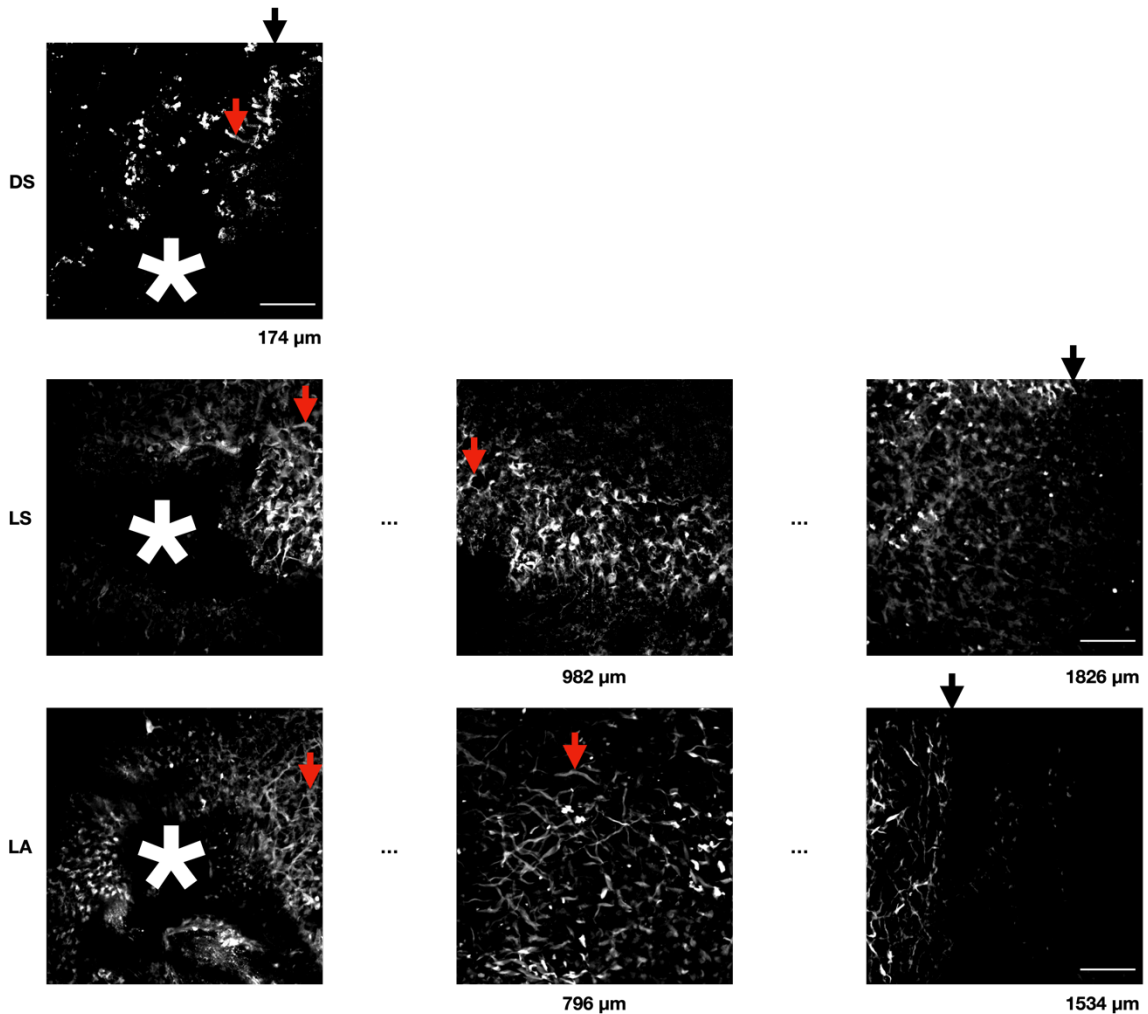
Confocal photomicrographs showing the effect of 1 μM KT 5823 on Neurobiotin spread after stab loading in retinas subject to dark-suppression (DS), light-sensitization (LS), and light-adaptation (LA) (location of stab indicated by large white asterisk). The left panel shows the region where the stab was made. The spread of Neurobiotin is illustrated by a panel (left) at the stab, a panel (middle) at a retinal location distant from the stab (distance indicated by the value, centered and below the panel) and a third (right) panel where Neurobiotin spread ended. Distance values placed below and to the right of a panel indicates the complete distance of Neurobiotin spread from the edge of the stab. The black arrow indicates the location where Neurobiotin spread was found to end in the image. Red arrows indicate the location of presumptive HATs. Scale bars = 100 μm.

Figure 3.6. Effect of KT5823 under differing light conditions and at varying concentrations on mean Neurobiotin spread in isolated goldfish retina after stab loading.

A) Mean distance ± standard deviation of Neurobiotin spread in μm from the stab point to the farthest point where labelling was detected. DS=dark-suppression, LS=light-sensitization, and LA=light-adaptation. Checkerboard bars indicate control conditions (same data as in Fig. 2.1) and filled bars indicate the same light conditions but in the presence of 1 μM KT 5823. * indicates $p < 0.05$. B) Mean distance ± standard deviation of the effect of different concentrations of KT 5823 on Neurobiotin spread in light-adapted retinas. The checkerboard bar indicates control LA conditions (same data as in Fig. 2.1) and filled bars indicate the same light condition (LA) but in the presence of different concentrations of KT 5823. Values provided above each bar indicate sample size.

A
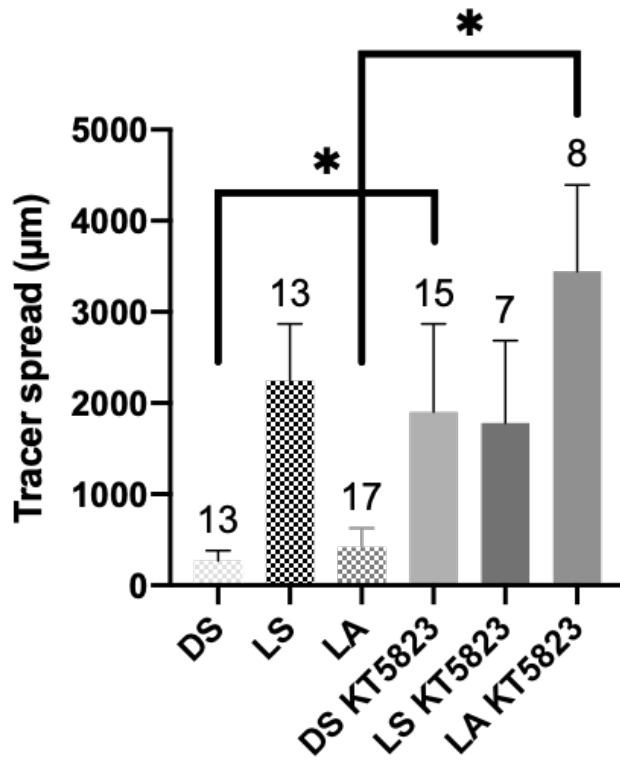
B
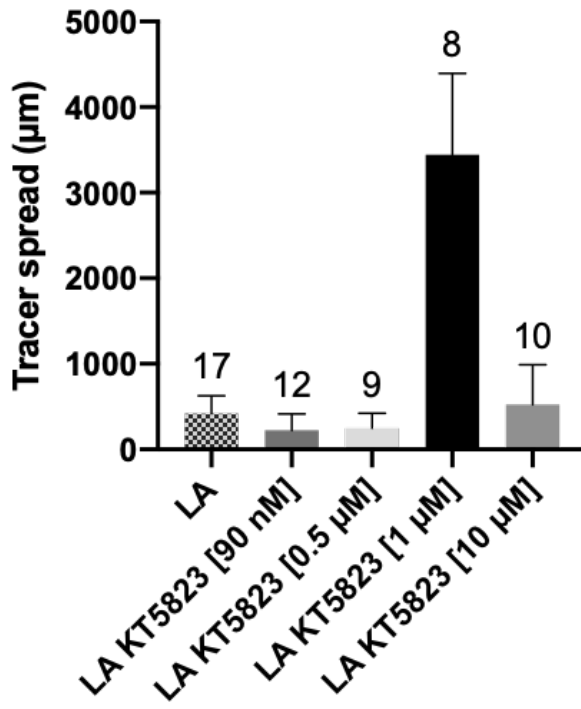
Figure 3.7. Effect of dbcAMP on Neurobiotin spread in isolated light-sensitized goldfish retina after stab loading.

A) Confocal photomicrographs showing the effect of 1000 µM (left) and 10 µM (right) dbcAMP on Neurobiotin spread after stab loading in light-sensitized (LS) retina (location of stab indicated by large white asterisk). The black arrows indicate the location where Neurobiotin spread was found to end. Distance values at the lower right corner indicate the extent of Neurobiotin spread. Red arrows indicate the location of presumptive HATs. Scale bars = 100 µm. B) Mean distance ± standard deviation of Neurobiotin spread in µm from the stab point to the farthest point where labelling was detected. The checkerboard bar indicates control LS (same data as in Fig. 2.1) and filled bars indicate the same light condition (LS) but in the presence of 1-1000 µM dbcAMP. Values provided above each bar indicate sample size. ** indicates $p < 0.005$ and **** indicates $p < 0.0001$.
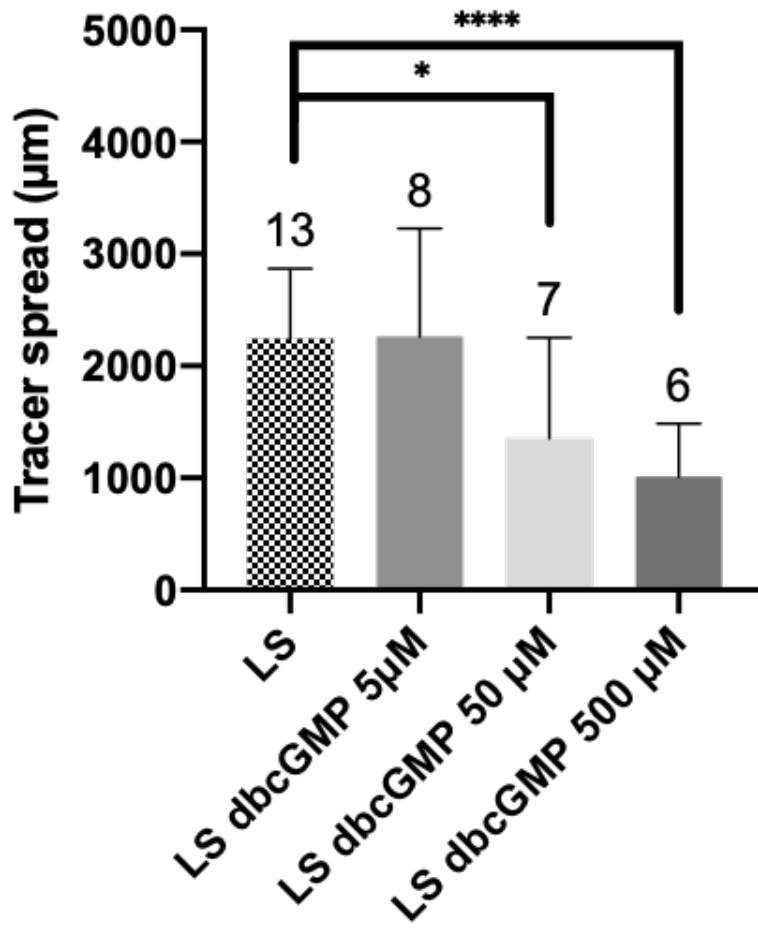
A

LS
cAMP
1000

126 µm

LS
cAMP
10

282 µm
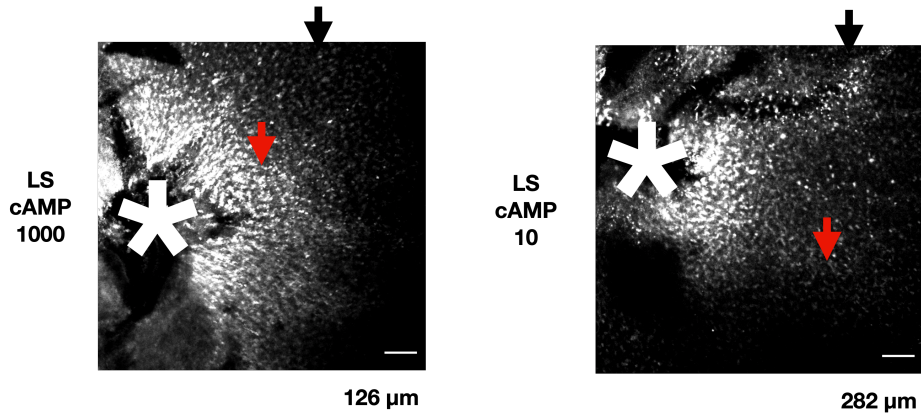
B
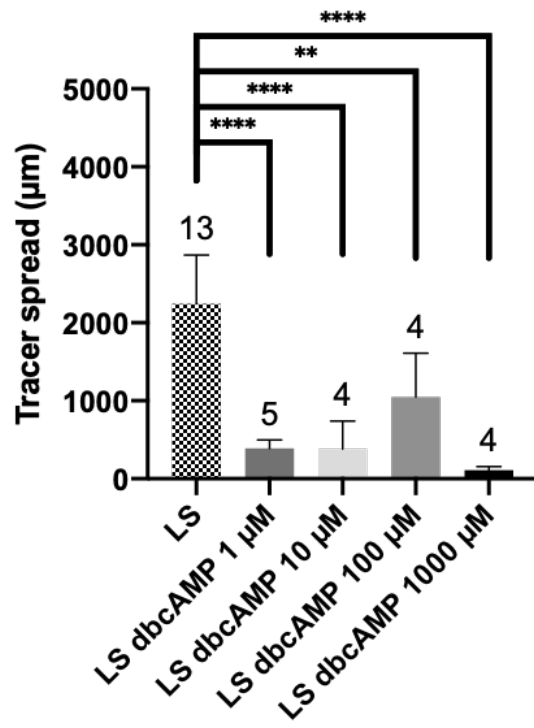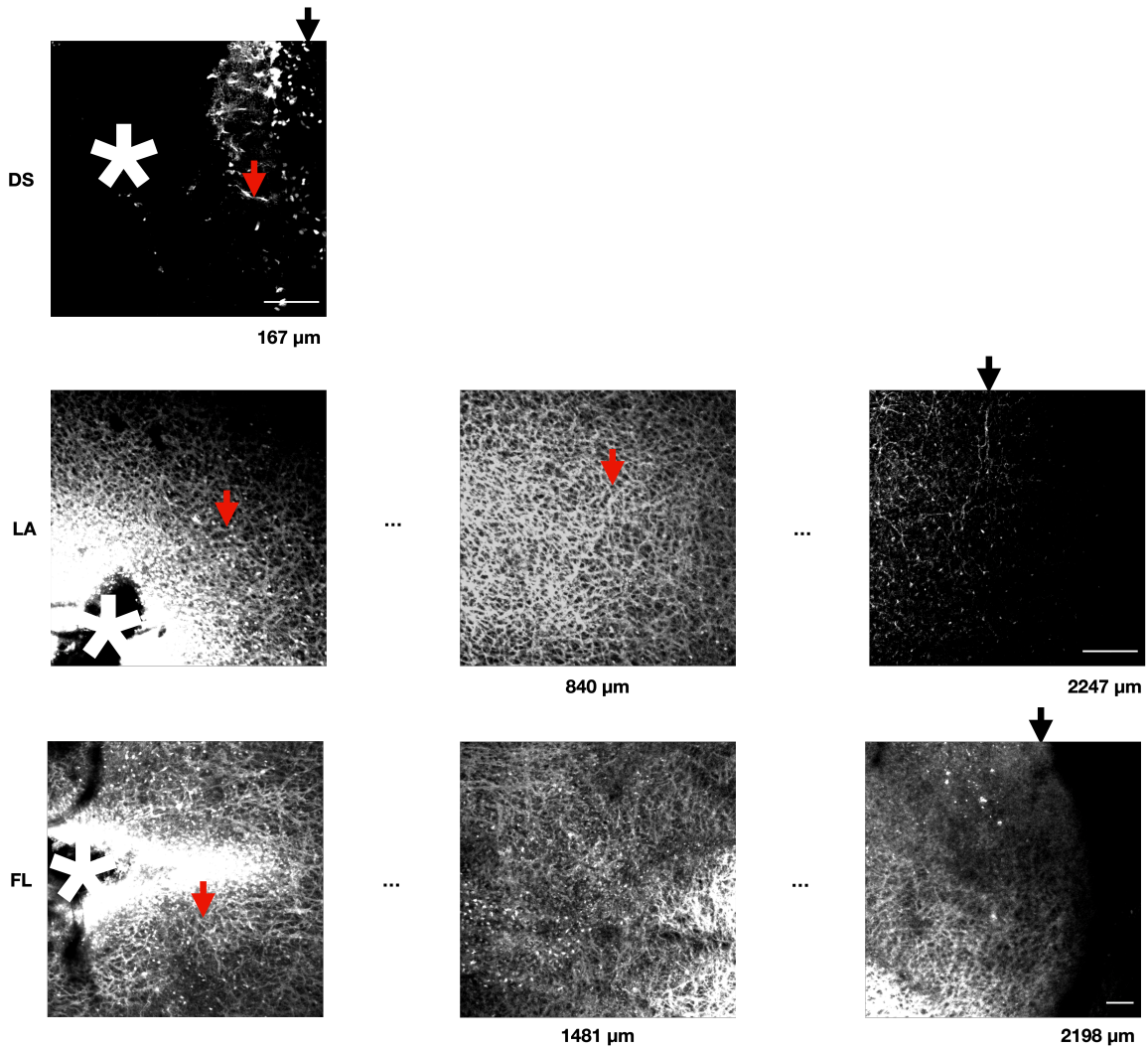
Figure 3.8. Effect of H89 on Neurobiotin spread after stab loading under differing light conditions in isolated goldfish retina.

Confocal photomicrographs showing the effect of 0.1 µM H89 on Neurobiotin spread after stab loading in retinas subject to dark-suppression (DS), light-adaptation (LA) and flickering light (FL) (location of stab indicated by large white asterisk). The left panel always shows the region where the stab was made. If Neurobiotin spread extended beyond the region depicted in the left panel, additional panels are added. In the case of three panels the spread of Neurobiotin is illustrated by a panel (left) at the stab, a panel (middle) at a retinal location distant from the stab (distance indicated by the value, centered and below the panel) and a third (right) panel where Neurobiotin spread ended. Distance values placed below and to the right of a panel indicates the complete distance of Neurobiotin spread from the edge of the stab. The black arrow indicates the location where Neurobiotin spread was found to end in the image. Red arrows indicate the location of presumptive HATs. Scale bars = 100 µm.

Figure 3.9. Effect of H89 on mean Neurobiotin spread after stab loading in isolated goldfish retina under different light conditions and the effect of KT5823 and L-NAME on mean Neurobiotin spread under flickering light conditions.

Mean distance ± standard deviation of Neurobiotin spread in μm from the stab point to the farthest detectable labelling under four light conditions: Dark-suppression (DS), light-adaptation (LA), and flickering light (FL). Also shown are the effects of 1 μM KT 5823 and 1.0 mM L-NAME on mean Neurobiotin spread under flickering light conditions. Checkerboard bars indicate control conditions (same data as in Fig. 2.1). Values provided above each bar indicate sample size. **** indicates $p < 0.0001$.

CHAPTER 4:
ROLE OF PKG IN HORIZONTAL CELL SOMATA LIGHT-ADAPTATION

4.1 Introduction

In the previous two chapters, the modulation of gap junction coupling between goldfish horizontal cell axon terminals (HATs) was studied using the spread of stab loaded Neurobiotin as an indicator of gap junction coupling. These experiments were valuable for two reasons: 1) they established, for the first time, that HAT gap junction coupling between HATs can be modulated by light conditions and by drugs known to affect HCS gap junction coupling; 2) they demonstrated that nitric oxide (NO) contributes to the reduction of gap junction coupling between HATs after exposure to bright light (light-adaptation), a mechanism also implicated in the reduction of horizontal cell somata (HCS) receptive-field size following light-adaptation (Daniels and Baldridge, 2011). As demonstrated previously in HCS (Pottek et al., 1997), results from Chapter 3 also showed that a membrane-permeable analogue of cyclic GMP reduced HAT gap junction coupling, consistent with an effect of NO mediated by soluble guanylyl cyclase and cyclic GMP (Garthwaite, 1991). I also showed that a protein kinase G (PKG) inhibitor, KT5823, blocked the effect of light-adaptation on the spread of Neurobiotin between HATs under control conditions, consistent with modulation of HAT gap junction permeability by PKG.

To extend the studies in this thesis to include HCS, I first used intracellular recording of goldfish HCS to assess the effect of KT5823 on receptive-field size. To place this in context, the effect of light conditions (light-sensitization and light-adaptation) and membrane-permeable cyclic GMP on HCS receptive-field size was also assessed. Seeking to extend the study of HCS gap junction coupling beyond the fish

retina, I utilized "cut loading" of Neurobiotin (Choi et al., 2012) to study HCS gap junction coupling in the mouse retina.

The mouse retina contains a single type of HCS (an axon bearing B-type cell) (Peichl and González-Soriano, 1994; Masuda et al., 2014) that contacts cones via neurites from the soma and contacts rods via the axon terminal (Peichl and González-Soriano, 1994). Although it was previously thought that the soma and axon terminal were electrically isolated by the axon (Nelson et al., 1975) another study demonstrated transmission from the soma to the axon terminal, but not from the terminal to the soma (Trümpler et al., 2008). Mouse horizontal cells possess gap junctions at the level of the soma (between neurites and axons) composed of connexin57 (Hombach et al., 2004; Janssen-Bienhold et al., 2009) but at axon terminals gap junctions are composed of connexin50 (Dorgau et al., 2015). There are few prior studies of HCS gap junction coupling in the mouse retina. The first neuromodulator shown to affect mouse HCS gap junction coupling was retinoic acid (Weiler et al., 1999). This study employed Neurobiotin spread after intracellular injection into HCS and reported that retinoic acid reduced coupling. This was followed by a similar study that examined the effect of dopamine (He et al., 2000) and showed that it reduced Neurobiotin tracer coupling. The effect of NO on mouse HCS gap junction coupling has not been examined, nor the effect of cyclic GMP or PKG. In addition, it has not been demonstrated if altered light conditions (exposure to dim versus bright light) can modulate HCS gap junction coupling in the mouse.

Therefore, using cut loading of Neurobiotin in isolated mouse retina preparations, I evaluated gap junction coupling between HCS and examined the effect of dim and bright

light exposure, and the effect of the NO donor DETA NONOate or dopamine on

Neurobiotin spread in retinas maintained under dim light conditions. I also examined the

effect of the PKG inhibitor KT5823 on tracer spread in retinas exposed to bright light.

The terminology used to describe triphasic adaptation in the fish retina may or may

not be applicable to mouse HC. Therefore, rather than describing light conditions as

being light-sensitized or light-adapted, for the study of mouse HCS we will characterize

light exposure as being to either dim light or bright light.

4.2 Methods

4.2.1 Intracellular recording of goldfish HCS

The isolated goldfish retina preparation was prepared as described in Chapter 2

(Section 2.2.1) with the only difference being that after isolation, retinas were removed

from the filter paper and placed into a chamber and constantly superfused with Ringer's

solution (bubbled with 95% O_2, 5% CO_2, pH 7.4). The preparation was superfused in

darkness for 15-20 min before HCS recording was initiated. Borosilicate glass electrodes

were pulled on a Flaming–Brown model P-97 microelectrode puller (Sutter Instruments,

Novato, CA) and filled with 2.5 M KCl, yielding a resistance of 50–150 MΩ (measured

using the intracellular amplifier, see below). Light stimulus was produced using an X-

Cite Series 120Q lamp (Excelitas, Waltham, MA) with both a 400 nm longpass and

infrared block (shortpass 800 nm) filter in the light path. The maximum unattenuated

light irradiance (I_0) was 250 µW/cm2 that was adjusted by placing calibrated neutral

density filters in the light path.

Electrodes were positioned into the retina using a micropositioning system (MP-

225; Sutter Instruments). Intracellular recordings were amplified using a MultiClamp

700A amplifier (Molecular Devices, San Jose, CA) and recorded and analyzed using Axoscope 9.0 (Axon Instruments Inc., Union City, CA). HCS were encountered 30–50 μm below the surface of the retina and were identified from their characteristic hyperpolarizing responses to broad spectrum (white) light. Different subtypes of HCS were not distinguished. The receptive field size of HCS were assessed by comparing the peak response to a visually centred (to the recording electrode) 1.8 mm diameter spot of white light to that of an annulus (outer diameter 3.5 mm and inner diameter 2.9 mm) with both at an intensity of -2.5 log units attenuated from I_0 by a neutral density filter. Stimulus duration was 500 msec.

Responses to spot and annulus stimuli were recorded from separte cells under one of six different conditions: 1) untreated, under the initial conditions in Ringer alone; 2) after exposure to full-field illumination (-1.0 log unit attenuated from I_0) for 1 min; 3) in Ringer containing 500 μM dibutyryl cyclic GMP (dbcGMP); 4) in Ringer containing 500 μM dbcGMP and exposed to full-field illumination; 5) in Ringer containing 1 μM KT5823; 6) in Ringer containing 1 μM KT5823 and exposed to full-field illumination. For detail about the drugs see Chapter 3 (Table 3.1, Section 3.2.2, 3.2.3).

The response to the annulus relative to the spot was quantified by dividing the amplitude of the response to the annulus by the amplitude of the response to the spot to produce an annulus/spot ratio (A/S ratio). The relative response to spot and annulus stimuli (and ratio) has been used extensiveli as an indicator of HCS receptive field size (Baldridge and Ball, 1991; Pottek et al., 1997; Djamgoz et al., 1998; Weiler et al., 1998; Pottek and Weiler, 2000; Xin and Bloomfield, 2000; Furukawa et al., 2002). Statistical comparisons were done using analysis of variance with the Bonferroni post-test for

multiple comparisons (Prism, GraphPad, San Diego, CA), and data are reported as mean ± standard deviation.

4.2.2 Cut loading of HCS in the mouse retina

All experiments were conducted in accordance with the Dalhousie University Committee on Laboratory Animals. Cut loading of mouse retina followed the method described by Choi et al. (2012). C57BL/7 mice (Jackson Laboratory, Bar Harbor, ME) were housed in a standard 12 hr light–dark cycle and fed ad libitum. Animals were sacrificed 4-5 hours into the light cycle by intraperitoneal injection of sodium pentobarbital (100 mg/kg, Euthansol 240 mg/mL; CDMV, Saint-Hyacinthe, QC, Canada). Eyes were enucleated and placed in room temperature Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich Corp., Oakville, ON, Canada) buffered with 10 mM HEPES and bubbled with 100% O_2. Retinas were dissected and mounted on filter paper photoreceptor side facing up and maintained under dim red light (~4 lux) for 10 min.

Retinas were then subject to one of the 5 conditions, in each case for 15 min: 1) maintained under dim red light in HBSS; 2) maintained under dim red light in Ringer containing the NO donor DETANONOate (100 µM); 3) maintained under dim red light in Ringer containing dopamine (20 µM); 4) exposed to bright light (~200 lux); 5) exposed to bright light in Ringer containing 1 µM KT5823. After the 15 min treatment period, Neurobiotin was applied. A #11 scalpel blade was dipped in 5% Neurobiotin (dissolved in water) and then used to make a cut across the retina. After application of Neurobiotin retinas were maintained for an additional 30 min under the same light or drug condition and then fixed in 2% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) for 10 min, rinsed and washed overnight in PB, and then washed again three times

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for 7 min in PB. Retinas were then incubated for 2 days in 10% Streptavidin conjugated to Alexa Fluor 488 (ThermoFisher #S11223) and a calbindin monoclonal antibody (1:1000 Sigma-Aldrich, #C9848) in PB containing 0.3% Triton X-100 and 3% normal donkey serum (Millipore Sigma). Retinas were washed three times for 7 min with PB and then incubated for 2 days in Cy3 goat anti-mouse secondary (1:200 ThermoFisher #M30010) in phosphate buffer. Retinas were then washed three times for 7 min in PB before mounting on a slide using 50% glycerol in 0.1M phosphate buffer media. To ensure that the Cy3 goat anti-mouse secondary antibody did not label mouse HCS independent of the calbindin monoclonal antibody, a control experiment was performed where the secondary was applied but the primary antibody omitted. No labelling by the secondary alone could be detected.

Details of image acquisition and evaluation of Neurobiotin spread were also the same as for stab loading as was the statistics used to evaluate mean data. To visualize Cy3 fluorescence by confocal microscopy, a 543.5 nm laser was used for excitation coupled to a 586 ± 40 nm emission filter.

4.2.3 Statistical analysis

All data are presented as mean ± standard deviation (± SD). A one-way analysis of variance (ANOVA) and Bonferroni corrected for multiple comparisons was run once for electrophysiological data ($F = 10.91$, $p < 0.0001$) and once for mouse Neurobiotin cut loading data ($F = 160.2$, $p < 0.0001$) (Prism, GraphPad, San Diego, CA).

4.3 Results

4.3.1 Effect of dbcGMP and KT5823 on goldfish HCS receptive-field size

Examples of the responses of goldfish HCS to annulus and spot stimuli are shown in Fig. 4.1. In the recording from a HCS from a retina maintained under dim light (light-sensitized), the responses to the annulus and spot stimuli were approximately equivalent (Fig. 4.1, LS CTRL). In fact, in this cell the response to the annulus was somewhat greater than the response to the spot. A HCS recorded after bright light exposure (light-adaptation) is shown in Fig. 4.1 (LA CTRL) and shows a reduction of responsiveness and a reduction of the response to the annulus relative to the response to the spot. Similar results were achieved by adding 500 µM dbcGMP to the Ringer's solution under dim light with the response to the annulus decreased relative to the response to the spot (Fig. 4.1, LS cyclic GMP). Exposing retina to bright light (light-adaptation) in the presence of 500 µM dbcGMP resulted in relative responses to the annulus and spot (Fig. 4.1, LA cyclic GMP) like those obtained by light-adaptation or dbcGMP treatment of light-sensitized retina. Recordings from HCS in light-sensitized retinas superfused by Ringer containing the PKG inhibitor KT5823 (1 µM) resulted in comparable responses to annulus and spot stimuli (Fig. 4.1, LS+KT). However, the response to the annulus was similar to the response to the spot (despite reduced responsiveness) when the retina was exposed to bright light (light-adaptation) in the presence of KT5823-containing Ringer (LA+KT).

Similar results were obtained from all HCS recorded as expressed by mean values of the ratio of the annulus response/spot (A/S ratio) (Fig. 4.2). The mean A/S ratio for HCS in light-sensitized retinas under control conditions (Ringer only) was 1.35 ± 0.50 ($\bar{x}$

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± SD, n=7; Fig. 4.2, LS CTRL). Compared to light-sensitized retinas, light-adaptation

reduced (p<0.01) the mean A/S ratio (0.35 ± 0.31, n=4; Fig. 4.2, LA CTRL) as did

treatment with dbcGMP (p<0.001, 0.15 ± 0.20, n=5; Fig. 4.2, LS cyclic GMP). The

mean A/S ratio from retinas subject to bright light (light-adaptation) in Ringer containing

dbcGMP (0.43 ± 0.29, n=7; Fig. 4.2, LA cyclic GMP) was not different (p>0.05) from

the mean A/S ratio from light-adapted retinas superfused with control Ringer. The mean

A/S ratio for HCS from light-sensitized retinas in Ringer containing KT5823 (0.98 ±

0.18, n=8; Fig. 4.2, LS KT) was not different (p>0.05) from the A/S ratio in control (LS

CTRL) retinas, but mean A/S ratio from HCS recorded from light-adapted retinas

superfused with KT5823-containing Ringer (1.48 ± 0.68, n=8; Fig. 4.2, LA KT) was

different (p<0.001) from the mean A/S ratio of HCS recorded from control retinas (LA

CTRL).

4.3.2 Cut loading of HCS in mouse retina with Neurobiotin

Unlike the experience with goldfish retina (see Chapter 2), cut loading proved an

effective method to load HCS with Neurobiotin in the isolated mouse retina. Mice

possess a single type of axon-bearing horizontal cell that is also calbindin-

immunoreactive (IR) (Peichl and González-Soriano, 1994; Masuda et al., 2014).

Therefore, I was able to confirm the identity of the Neurobiotin-loaded cells as HCS by

performing calbindin immunohistochemistry. Figure 4.3A shows calbindin-

immunoreactivity (left) in a region of mouse retina also loaded with Neurobiotin (Fig.

4.3A, middle). There is clear evidence of colocalization of Neurobiotin and calbindin

(Fig. 4.3A, right).

4.3.3 Modulation of mouse HCS gap junction coupling

In retinas exposed to dim light conditions, Neurobiotin spread away from the edge of the cut into calbindin-IR HCS extending, in the image shown, 590 μm (Fig. 4.3B, DL CTRL). Although co-localization of Neurobiotin and calbindin-IR can be seen, revealing the spread of Neurobiotin between HCS, near where the Neurobiotin was loaded (the cut, left hand edge of image) there were also some Neurobiotin-labelled cells that were apparently not calbindin-IR (Fig. 4.3B, DL CTRL). In fact, these are cells where the level of Alexa 488 fluorescence (Neurobiotin was localized with Streptavidin conjugated to Alexa 488) was so great that their fluorescence signal overwhelmed the Cy3 fluorescence (of the secondary antibody used to reveal the presence of the calbindin primary antibody). Especially bright Neurobiotin/Alexa 488 labelling that overwhelmed the Cy3 signal is also seen in other images (Fig. 3.4B DL DETANONOate, BL KT5823). Retinas exposed to bright light, treated with the 100 μM DETA NONOate or 20 μM dopamine, showed less spread of Neurobiotin between HCS (Fig. 4.3B, BL CTRL, DL DETA NONOATE, DL DOPAMINE). In the images provided, Neurobiotin spread was 89 μm after treatment with DETA NONOate (Fig. 4.2B, DL DETA NONOate), 163 μm after treatment with dopamine (Fig. 4.3B, DL DOPAMINE), and 90 μm after exposure to bright light (Fig. 4.3B, BL). In most cases evidence of Neurobiotin could be seen immediately adjacent to the cut, even in cases where Neurobiotin did not spread much beyond the cut. Retinas exposed to bright light in the presence of the PKG inhibitor KT5823 (1 μM) showed spread of Neurobiotin within HCS (Fig. 4.3B, DL KT5823), in fact the spread was greater than in retinas exposed to dim light (Fig. 4.3B, DL CTRL). Because of the large extent of spread, the image of KT5823 was divided to show Neurobiotin spread and calbindin fluorescence at three locations: near the cut (left

panel), at the point where Neurobiotin spread ended (at 1203 µm, right panel) and at a point in between (middle panel, centred at 769 µm).

Similar results were obtained in all retinas studied (Fig. 4.4). Mean spread of Neurobiotin in retinas maintained under dim light was 525 ± 94 µm, ($\bar{x} \pm$ SD, n=7; Fig. 4.4, DL CTRL). Exposure to bright light reduced (p<0.0001) the mean spread of Neurobiotin (208 ± 54 µm, n=5; Fig. 4.4, BL CTRL) as did treatment with 100 µM DETA NONOate (p<0.0001; 68 ± 32 µm, n=5; Fig. 4.4, DL DETA NONOate) or 20 µM dopamine (p<0.0001; 160 ± 105 µm, n=7; Fig. 4.4, DL DOPAMINE). Retinas exposed to bright light in the presence of 1 µM KT5823 had expanded spread of Neurobiotin (1367 ± 158 µm, n=6; Fig. 4.4, BL KT5823) that was greater (p<0.0001) than mean Neurobiotin spread in control (Ringer only) retinas maintained under dim light.

4.4 Discussion

Chapter 2 and Chapter 3 of this thesis used stab loading of Neurobiotin to study the modulation of gap junction coupling between HATs in the goldfish retina. To extend these studies to HCS, I performed intracellular recording of HCS in isolated goldfish retina with the primary aim of examining the effect of the PKG inhibitor KT5823 on the receptive-field size of HCS following light-adaptation. To extend the study beyond fish, I employed cut loading of Neurobiotin in mouse retina and determined the effect of light exposure (dim versus bright light) and the effect of the NO donor (DETA NONOate) and dopamine on Neurobiotin spread between HCS in mouse retina maintained under dim light. I also tested the effect of KT5823 on Neurobiotin spread in mouse retinas exposed to bright light.

In this section I will discuss the interpretation of the results concerning the possible mechanism mediating light-adaptation of goldfish HCS as suggested by HCS receptive-field size. I will then compare and contrast the results from my study of mouse HCS with what is known from previous work regarding the modulation gap junction coupling of mouse HCS. Additional discussion of the results in this chapter, in context with the studies presented in Chapter 2 and 3, will be considered in Chapter 5.

4.4.1 Summary of results

The receptive-field size of HCS in goldfish retina was assessed by comparing the response of HCS in isolated retina preparations to annulus and spot stimuli and calculating the ratio of the annulus response/spot response (A/S ratio). Under conditions where retinas were maintained under dim light conditions (light-sensitized), the annulus response was similar or greater than the response to the spot, resulting in A/S ratios ≥ 1.0. When such light-sensitized retinas were exposed to bright light (light-adaptation) the response to the annulus decreased relative to the response to the spot (A/S ratio < 1.0). Application of the membrane-permeable analogue of cyclic GMP, dibutyryl cyclic GMP (dbcGMP) to light-sensitized retinas also reduced the A/S ratio but did not alter A/S ratio after light-adaptation. The PKG inhibitor, KT5823, had no effect on the A/S ratio of HCS in light-sensitized retina but blocked the decrease of A/S ratio produced by light-adaptation.

In the isolated mouse retina, cut loading of Neurobiotin was an effective means of loading HCS with Neurobiotin. Calbindin immunohistochemistry was used to confirm the identify of mouse HCS. Under dim light conditions Neurobiotin spread ~500 μm from the cut site. In retinas exposed to bright light Neurobiotin was restricted to near the

cut site. Similar results were obtained when retinas maintained under dim light were treated with the NO donor DETA NONOate or dopamine. In retinas exposed to bright light in the presence of the PKG inhibitor KT5823, Neurobiotin spread was increased, not only reaching the same extent of spread as in retinas maintained under dim light conditions but extending even further (> 1,000 μm).

4.4.2 Goldfish HCS receptive-field size

Gap junction coupling between horizontal cells allows for a receptive-field size larger than the region over which they receive synaptic input from photoreceptors (Naka and Rushton, 1967). Although the receptive-field size can be measured by assessing the responses of HCS to stimuli translated across the receptive-field (Daniels and Baldridge, 2011) a more common approach is to compare the response to stimuli displaced from the cell being recorded (e.g. an annulus) to the response to a spot centred over the cell being recorded (Baldridge and Ball, 1991; Pottek et al., 1997; Djamgoz et al., 1998; Weiler et al., 1998; Weiler et al., 2000). The extent that altered electrical currents generated by the displaced stimulus influence a HCS being recorded is dependent on the presence of gap junction coupling. Gap junction coupling can also affect the responses to the centred spot stimuli. If small (e.g. ≤ 0.5 mm) the response can increase when HCS gap junctions are uncoupled due to increased input resistance; for larger spots, the response can be enhanced by gap junctions due to spatial summation, meaning that responses would decrease when gap junctions are uncoupled; for very large spots (or full-field stimuli) changing gap junction coupling may have little or no influence on response amplitude as, under these conditions, HCS will be equipotential, all receiving the same level of synaptic input. The stimuli I used was a moderately sized spot (1.8 mm diameter) and an even

larger annulus (2.9 mm inner diameter, 3.5 mm outer diameter). The annulus and the

spot would stimulate approximately the same spatial extent of the retina (spot 2.6 mm^2;

annulus 3.0 mm^2) meaning that if gap junction coupling is robust then the response to the

annulus and spot should be similar (or, given the slightly larger field stimulated by the

annulus, the annulus response could be somewhat greater). In fact, the average response

to the annulus employed here was somewhat greater than the response to the spot (A/S

ratio > 1.0). Therefore, the A/S ratio values reported here suggest increased receptive-

field size when A/S ratios were increased (> 1.0) and decreased receptive-field size when

the A/S ratio was decreased (< 1.0).

The changes in A/S ratio, and therefore receptive-field size, imply changes in gap

junction coupling (increased coupling when A/S ratio increases, decreased coupling when

A/S ratio decreases). However, changes in the size of the receptive-field size can also be

affected by changes in HCS membrane resistance. As described in Section 1.12, the

spread of electrical current within the network of gap junction coupled HCS can be

modelled using cable theory (Lamb, 1976). This means that current spread within

coupled HCS is a function not only of the internal resistance of the network (R_i), the most

significant component of which is the resistance of the gap junctions, but also membrane

resistance (R_m). Decreased R_m would mean more current passes across the membrane

rather than passing longitudinally within HCS network thereby resulting in a reduction of

receptive-field size. Although there is no evidence of a neuromodulator that modulates

HCS receptive-field size by only affecting R_m and not R_i, effects on R_m cannot be ruled

out as a contributing factor. Therefore, direct assessment of gap junction permeability,

using tracers like Neurobiotin, are essential to confirm that changes in receptive-field size involve changes in gap junction resistance.

In this study I did not determine the subtype of HCS being recorded (Section 1.5). Previous work has shown that the most common HCS impaled during intracellular recording in goldfish are the H2 subtype (Baldridge and Ball, 1991). But more important, this same study found no difference between the different types of HCS with respect to the effect of light-adaptation on receptive-field size. Therefore, in the present work we did not determine the separate HCS types during intracellular recording. The light responses of the HATs are indistinguishable from those recorded from the HCS to which they connect (Weiler and Zettler, 1979; Yagi, 1986). Although this makes it impossible to differentiate recordings from HATs versus HCS, in the present study I limited my search for HCS to the first 30-50 µm from the point of contact with the retina, making it unlikely that HATs were encountered.

The effect of light-adaptation on goldfish HCS receptive-field size reported here is similar to the findings of previous studies that showed increased receptive-field size in retinas exposed to dim light (light-sensitization) and a decrease of receptive-field size after exposure to bright light (light-adaptation) (Baldridge and Ball, 1991; Umino et al., 1991; Daniels and Baldridge, 2011). I also confirmed that HCS receptive-field size was reduced by treatment with the membrane-permeable analog of cyclic GMP, dbcGMP, applied by superfusion onto retinas maintained under dim light conditions (light-sensitized). This result agrees with previous studies of fish (carp) HCS that used a different cyclic GMP analogue, 8-bromo-cyclic AMP (Pottek et al., 1997). Lastly, prompted by the effect of the PKG inhibitor, KT5823, on Neurobiotin spread in HATs

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(Chapter 3) I tested the effect of this drug on HCS receptive-field size in retinas exposed to bright light (light-adaptation). KT5823 blocked the effect of light-adaptation, resulting in an expanded receptive-field size compared to the receptive-field size of HCS in light-adapted retinas. This result, and the effect of dbcGMP, are consistent with a mechanism whereby, during light-adaptation, elevated cyclic GMP stimulates PKG and PKG then phosphorylates gap junction connexins (Lampe and Lau, 2000; Warn-Cramer and Lau, 2004; Patel et al., 2006; Moreno and Lau, 2007). Considered together with the data showing that NO is involved with the reduction of receptive-field size in HCS (Daniels and Baldridge, 2011) this adds to the evidence that the mechanism of HCS light-adaptation involves NO, cyclic GMP and PKG. However, in Chapter 3 I found that the reduction of Neurobiotin spread within HATs in light-adapted retinas was reduced not only by KT5823 but also the PKA>PKG inhibitor H89. I did not examine the effect of H89 on HCS receptive-field size but the possible role of PKA, and the specificity of protein kinase inhibitors, will be discussed further in Chapter 5.

4.4.3 Cut loading of Neurobiotin in mouse HCS

In this chapter I report that cut loading of Neurobiotin in the isolated mouse retina is an effective means with which to load HCS. It was possible to definitively identify the cells loaded with Neurobiotin as HCS using immunohistochemistry for calbindin, a known marker of HCS in the mouse retina (Peichl and González-Soriano, 1994; Masuda et al., 2014). Under dim light conditions, Neurobiotin spread away from the cut site extending ~500 μm. The extent of spread by cut loading was similar to that observed in a study where Neurobiotin was injected intracellularly into mouse HCS (Weiler et al., 1999). However, in another study (He et al., 2000), also using intracellular injection, the

Neurobiotin spread was less (~150 µm). Nonetheless, He et al. (2000) showed that

application of dopamine reduced the spread of Neurobiotin between HCS. When

dopamine was applied to retinas maintained under dim light conditions I found no spread

of Neurobiotin beyond the edge of the cut where a small number of calbindin-IR HCS

could be seen loaded with Neurobiotin. A similar result was obtained when retinas (again

maintained under dim light conditions) were treated with the NO donor DETA NONOate.

In addition, I showed that exposing mouse retinas to bright light abolished the spread of

Neurobiotin within calbindin-IR HCS but that the effect of bright light could be blocked

by treatment with the PKG inhibitor KT5823. In fact, the spread of Neurobiotin within

calbindin-IR HCS was even greater in KT5823-treated retinas than under dim light

conditions.

These data are the first to report the effects of dim versus bright light, NO donor

and a PKG inhibitor on Neurobiotin spread in mouse HCS. Taken together they suggest

that gap junction coupling between mouse HCS is reduced, relative to dim light

conditions, by bright light, that NO can uncouple mouse HCS, and that PKG is involved

in the modulation of mouse HCS gap junction coupling during bright light exposure. A

role for PKG implies the possible action of NO, but this would need to be proven by, for

example, examining the impact of a nitric oxide synthase inhibitor on the effect of bright

light. That treatment with KT5823 resulted in Neurobiotin spread even more than that

seen under dim light conditions could suggest a degree of tonic activation of PKG,

perhaps due to increased NO, even under dim light conditions.

Published work (Weiler et al., 1999; He et al., 2000) and the results in this

chapter, suggest that the neuromodulation of mouse HCS gap junctions is similar in many

ways to what has been described in the fish. All three of the neuromodulators shown to uncouple fish HCS gap junctions (dopamine, retinoic acid and nitric oxide) also uncouple HCS gap junctions in the mouse and cyclic AMP and cyclic GMP analogues reduce HCS gap junction coupling in both fish and mice. Also, like fish, coupling is greater in mouse HCS maintained under dim light than after exposure to bright light and the PKG inhibitor KT5823 interferes with the reduction of fish HCS receptive-field size produced by light-adaptation and the reduction of tracer coupling in mouse HCS following bright light exposure. What has not been demonstrated in mice, but is known to some degree from studies of fish HCS, are the effects of prolonged darkness or flickering light on HCS gap junction coupling and the possible mechanisms involved. Specifically, it remains to be established if the reduction of HCS gap junction coupling in mice by bright light involves dopamine, as this has been shown not to be the case in fish (Baldridge and Ball, 1991; Umino et al., 1991). An outstanding question for both fish and mouse HCS is what role retinoic acid might play in the modulation of HCS gap junction coupling during different light conditions.
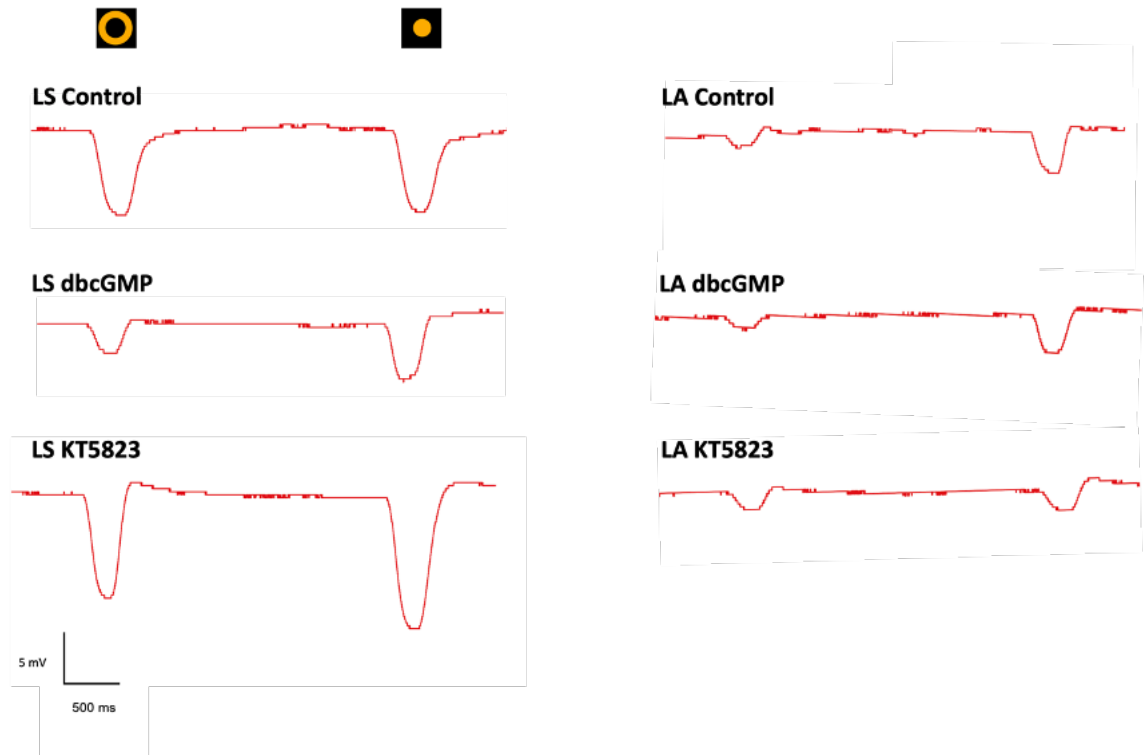
Figure 4.1. Intracellular recordings showing the effect of dbcGMP and KT5823 on responses of goldfish horizontal cells to annulus and spot stimuli under light-sensitized and light-adapted conditions.

Traces show responses of six different horizontal cells stimulated with an annulus (left) and spot (right) under two different light conditions (light-sensitized, LS, or light-adapted, LA) and under control (no drug) conditions or after treatment with 500 µM dbcGMP or 1 µM KT 5823.
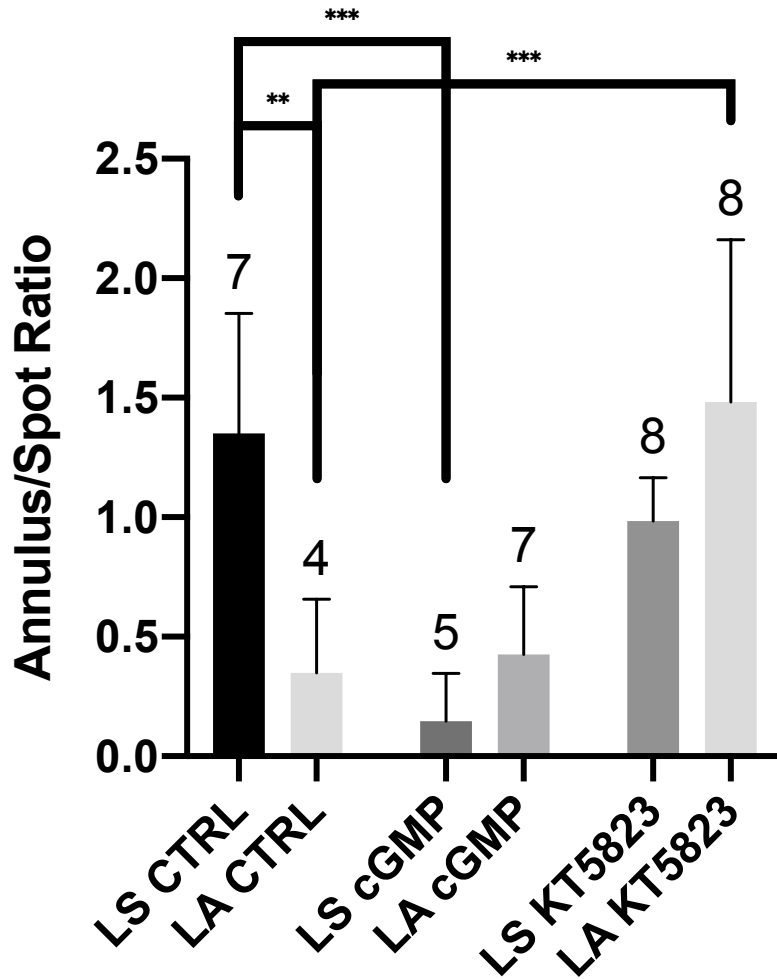
Figure 4.2. Effect of dbcGMP and KT5823 on horizontal cell annulus response/spot response ratio under light-sensitized and light-adapted conditions.

Mean annulus/spot ratio ± standard deviation of horizontal cells recorded from light-sensitized (LS), or light-adapted (LA) retinas under control (no drug) conditions or after treatment with 500 μM dbcGMP (cGMP) or 1 μM KT 5823 (KT). Values provided above each bar indicate sample size. ** = $p < 0.01$, *** = $p < 0.005$.

Figure 4.3. Effects of light conditions, KT5823, DETA NONOate, and dopamine on Neurobiotin spread in horizontal cells of isolated mouse retina after cut loading.

A) Confocal photomicrographs showing a region of flat mounted mouse retina subject to calbindin immunolabelling (red, left), Neurobiotin cut loading (green, middle), and the merge of the two images (right). B) Confocal micrographs showing the spread of cut loaded Neurobiotin in mouse horizontal cells, double labelled by calbindin immunohistochemistry. The location where Neurobiotin was applied is always at the left edge of the images. Neurobiotin spread was examined under control dim light (DL CTRL) or bright light (BL CTRL) conditions, or dim light conditions and treated with 100 µM DETA NONOate (DL DETA NONOate) or 20 µM dopamine (DL DOPAMINE), or bright light conditions and treated with 1 µM KT 5823 (BL KT5823). Neurobiotin spread following KT5823 treatment was extensive, so the labelling is represented by three panels: a panel (left) containing the location of the cut loading, a panel (middle) at a retinal location distant from the cut (distance indicated by the value, centered and below the panel) and a third (right) panel where Neurobiotin spread ended. Distance values placed below and to the right of all the images indicates the complete distance of Neurobiotin spread from the edge of the cut. Scale bar = 100 µm.

A

CALBINDIN   NEUROBIOTIN   MERGE

B

DL CTRL

590 µm

DL DETA NONOATE

89 µm

DL DOPAMINE

163 µm

BL CTRL

90 µm

BL KT5823

769 µm   1203 µm

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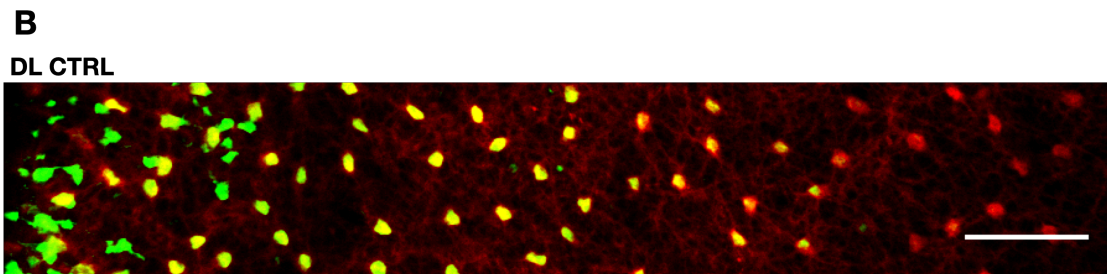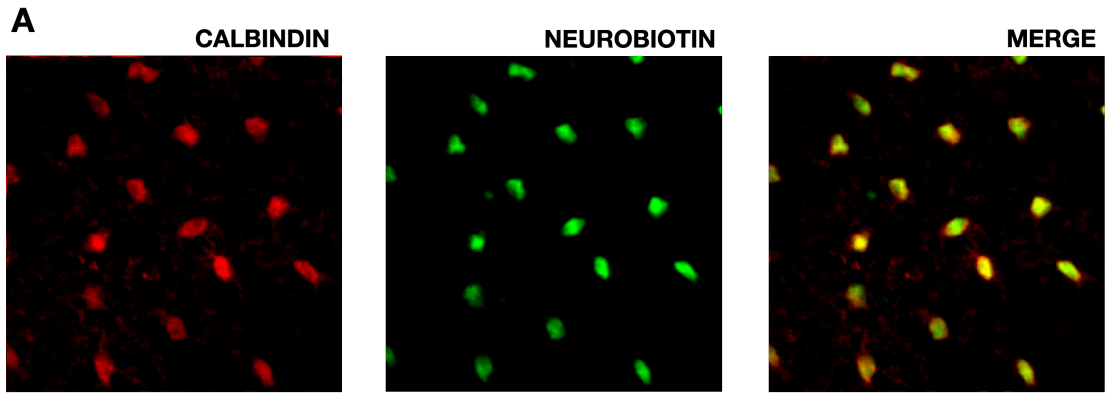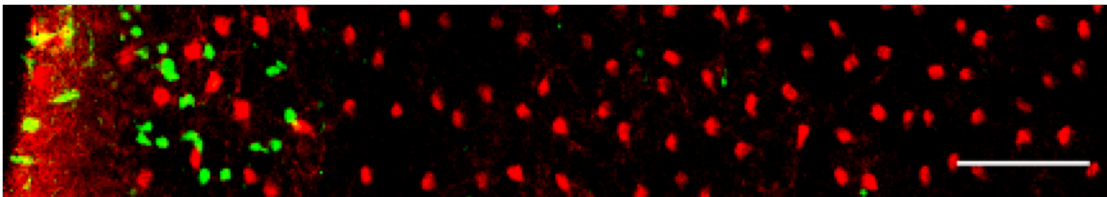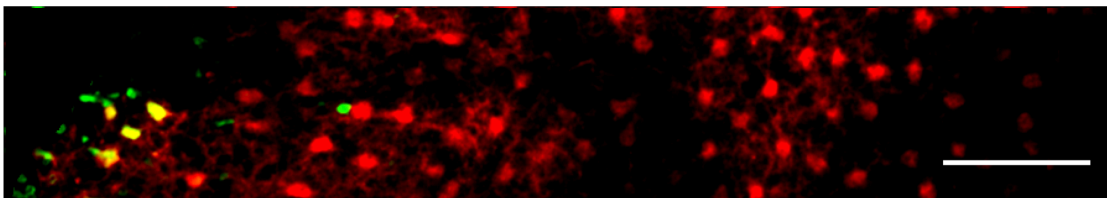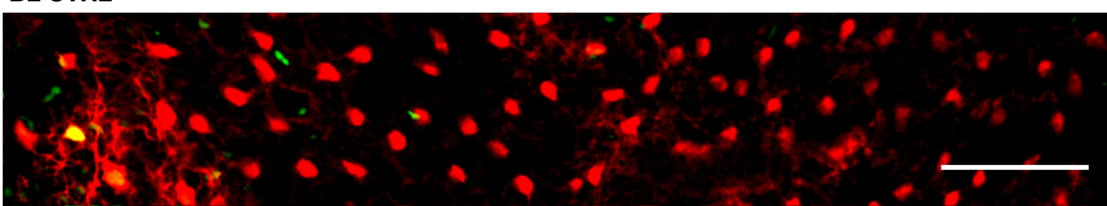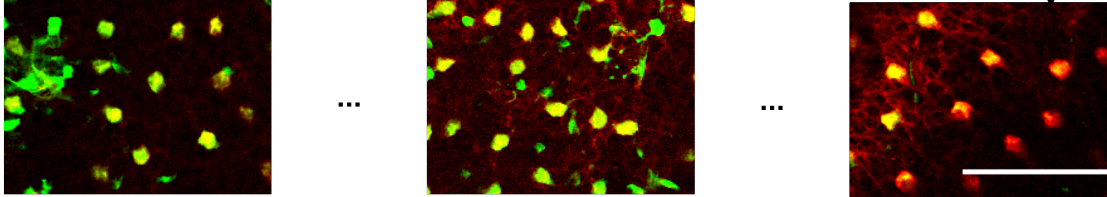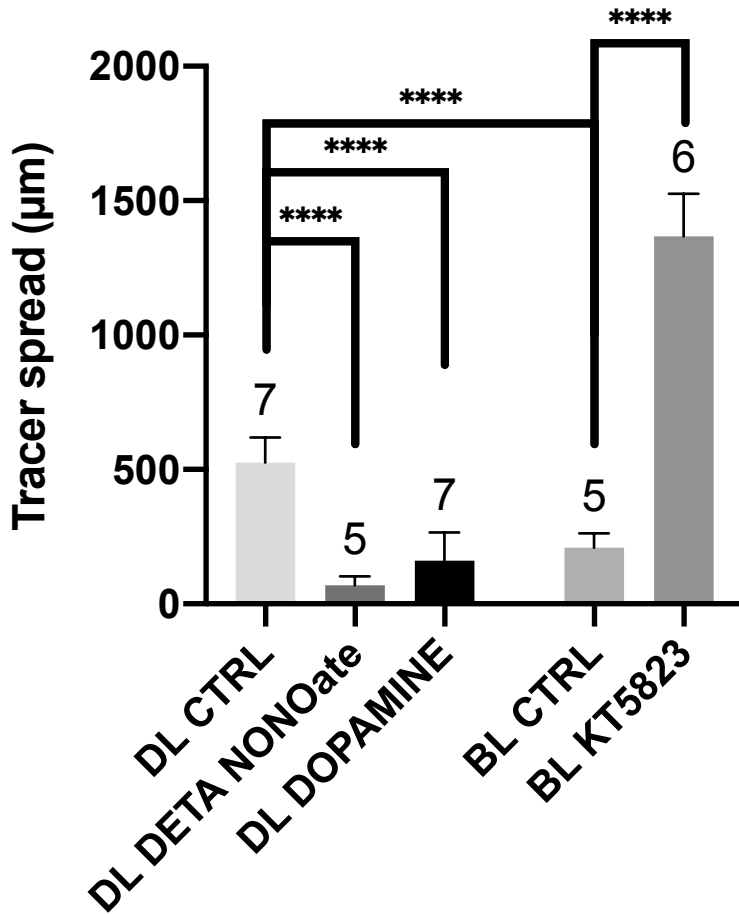
Figure 4.4. Effects of light conditions, KT5823, DETA NONOate, and dopamine on mean Neurobiotin spread in isolated mouse retina after cut loading.

Mean distance ± standard deviation of Neurobiotin spread in μm from the cut point to the farthest detectable point of labelling under control (CTRL) dim light (DL) or bright light (BL) conditions or after treatment (DL conditions) with 100 μM DETA NONOate or 20 μM dopamine or (BL conditions) with 1 μM KT 5823. Values provided above each bar indicate sample size. **** indicates $p < 0.0001$

CHAPTER 5:
GENERAL DISCUSSION

5.1 Preamble

The study of horizontal cells in the vertebrate retina has a long history, dating back at least to the time of Ramon y Cajal, but the start of the modern era was the work of Svaetichin who was the first to record (Svaetichin, 1953) the light-evoked responses of horizontal cells in isolated fish retina (see Piccolino (1986)). Since that time enormous progress has been made in our understanding of the morphology and physiology of horizontal cells in a wide range of vertebrates, from fish to man (see Boije et al. (2016)). Landmark discoveries in the history of horizontal cell research were the description of the pattern of connectivity of horizontal cells with photoreceptors (Stell and Lightfoot, 1975), that horizontal cells provide negative feedback to photoreceptors (Baylor et al., 1971) and that horizontal cells are extensively coupled to one another via gap junctions (Yamada and Ishikawa, 1965; Naka and Rushton, 1967). What these all have in common is that they formed the foundation on which a model of the circuitry underlying center-surround receptive fields of bipolar and ganglion cells was built (Naka and Nye, 1971; Kaneko, 1973).

The focus of this thesis is the coupling of horizontal cells by gap junctions and specifically addressing the fact that horizontal cell gap junction coupling is not static but can be reduced by neuromodulators, specifically dopamine (Teranishi et al., 1983), nitric oxide (Pottek et al., 1997) or retinoic acid (Weiler et al., 1998), and also by the level of ambient illumination (Tornqvist et al., 1988; Baldridge and Ball, 1991; Umino et al., 1991). With respect to the latter, it has been suggested in fish and rabbit that horizontal cell gap junction coupling is decreased following darkness (Tornqvist et al., 1988; Bloomfield et al., 1997), or after exposure to bright steady or flickering light (Baldridge

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and Ball, 1991; Umino et al., 1991), relative to an intermediate condition where retinas were exposed only to dim or moderate levels of illumination. This led to a model that posited three different phases of horizontal cell adaptation (see Baldridge (2001)): dark-suppression, where horizontal cell coupling is reduced; light-sensitization, where coupling is relatively increased, and light-adaptation, where coupling is also decreased. The terminology "dark-suppression" was used first by Yang et al. (1988b, 1988a) because, a least in fish retina, in addition to decreasing gap junction coupling, darkness dramatically decreases the responsiveness of horizontal cells. Responsiveness is increased or "sensitized" by exposure to dim light (Yang et al., 1988b; Baldridge et al., 1995), hence the term light-sensitization. Lastly, light-adaptation was used to describe the reduction of horizontal cell receptive-field size by bright light (Baldridge and Ball, 1991).

The most recent area of investigation along these lines has been to determine which neuromodulator mediates the effects of ambient illumination. The current working model is that dopamine, acting via D1 dopamine receptors, mediates the effects of dark-suppression and flickering light (Bloomfield et al., 1997; Bloomfield and Völgyi, 2004) but not light-adaptation (Baldridge and Ball, 1991). Subsequently, Daniels and Baldridge (2011) showed that the effect of light-adaptation on fish horizontal cells could be reduced by treatment with the nitric oxide synthase inhibitor providing evidence that nitric oxide (NO) is involved with light-adaptation.

5.2 Thesis Objectives

The objective of this thesis was to study further the mechanism of horizontal cell light-adaptation in the goldfish retina, focussing on the role of NO and protein kinase G

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(PKG) on horizontal cell gap junction coupling. PKG is a common downstream target of

NO-induced (via soluble guanylyl cyclase) increases of cyclic GMP (Garthwaite, 1991)

that then could phosphorylate gap junction protein (e.g. Patel et al. (2006)). Given the

evidence suggesting that horizontal cell light-adaptation involves NO (Daniels and

Baldridge, 2011), I predicted that a PKG inhibitor would reduce or block the effect of

bright light (light-adaptation). A novel approach was planned, using "cut loading" of the

gap junction-permeable tracer Neurobiotin (Choi et al., 2012) to directly assess goldfish

horizontal cell gap junction permeability, and then to examine the effect of light

conditions and drugs to target NO and PKG and, for comparison, protein kinase A

(PKA). Additional experiments examined the effect of PKG inhibitor on goldfish

horizontal cell receptive-field size, using intracellular recording, and on Neurobiotin

tracer spread in mouse horizontal cells following cut loading. Again, the primary

hypothesis was that PKG inhibitor would reduce the effect of light-adaptation on the

reduction of horizontal cell receptive-field size in goldfish and the spread of Neurobiotin

between horizontal cells in the mouse.

5.3 Stab loading

Cut loading was not found to be an effective means by which to load horizontal

cells in the goldfish retina (but was effective in the mouse retina). An alternate method,

"stab loading," was used and proved more useful in goldfish but with an important

limitation: loading was achieved in horizontal cell axon terminals (HATs) but not

horizontal cell somata (HCS).

In goldfish there are three types of horizontal cell somata (HCS) that receive input

from cone photoreceptors and one type that receives input from rods. The cone driven

HCS give rise to an axonal process that then descends deeper into the proximal INL (Stell, 1975) where it forms a HAT that makes synaptic contacts with amacrine, bipolar and interplexiform cells (Dowling et al., 1966; Marc and Liu, 1984; Marshak and Dowling, 1987). The function of these contacts, and of HAT in general, is not known. It has been shown that the light responses of HATs are indistinguishable from the responses of the HCS to which they connect (Weiler and Zettler, 1979; Yagi, 1986). This is remarkable given that the HCS and HAT are connected by a small (0.5 µm diameter) axon ~200 µm long (Stell, 1975). From simple cable theory, it would seem that the electrical signal could not propagate from the HCS to the HAT. Weiler and Zettler (1979) calculated that the signal would decrease by about 95% and, therefore, proposed a regenerative mechanism must be present. An alternate explanation arose when it was discovered that HATs are coupled by gap junctions (Kaneko and Stuart, 1984; Shigematsu and Yamada, 1988). Yagi (1986) argued that coupling between HATs, and with adjacent axons, shortens the effective length of the axon allowing electrotonic current flow from HCS to HATs and vice versa.

Studies using stab loading of Neurobiotin in goldfish retina in this thesis were used to study gap junction coupling of HATs. As much less is known about the modulation of HAT coupling, additional experiments were conducted to determine to what extent changes of HAT coupling match changes of HCS coupling, with respect to light conditions and the effect of neuromodulators.

5.4 Interpretation of results

5.4.1 Horizontal cell light-adaptation

The primary focus of this thesis was to investigate further the proposed role of NO and PKG in the reduction of horizontal cell gap junction coupling during light-adaptation. I showed that light-adaptation reduced Neurobiotin spread of HATs in stab loaded goldfish retina. The effect of light-adaptation was mimicked by application of NO donor to light-sensitized retina and reduced by the nitric oxide synthase inhibitor L-NAME. Consistent with a role for NO acting via soluble guanylyl cyclase, a membrane permeable analogue of cyclic GMP also reduced Neurobiotin spread. Lastly, the PKG inhibitor KT5823 also reduced the effect of light-adaptation. These studies suggest that HAT gap junction coupling is reduced during light-adaptation by a mechanism that involves NO, cyclic GMP and PKG (see Fig. 5.1).

An additional experiment showed that light-adaptation was also reduced by the PKA>PKG inhibitor H89. This was not expected because it has been shown that light-adaptation of HCS does not involve dopamine (Baldridge and Ball, 1991; Umino et al., 1991) that acts on HCS via D1 dopamine receptors, adenylyl cyclase, cyclic AMP and ultimately, PKA (Lasater, 1987; Yang et al., 1988a; McMahon et al., 1989). Although this could represent an effect of H89 on PKG, or the presence of another neuromodulator acting during light-adaptation, another possibility is cross-talk between PKG and PKA (Patel et al., 2006).

Patel et al. (2006) studied the modulation of Cx35, cloned from perch (a fish from the Percidae family, but a homologue of mammalian Cx36) that is expressed throughout the central nervous system (Rash et al., 2000) including the retina (O'Brien et al., 1996;

Güldenagel et al., 2000; O'Brien et al., 2004). In retina, Cx35 (or in mammals Cx36) contributes to gap junctions mediating homologous coupling between photoreceptors, bipolar cells, amacrine cells and ganglion cells and heterologous coupling between AII amacrine cells and ON cone bipolar cells (Völgyi et al., 2013). Patel et al. (2006) expressed Cx35 in HeLa cells and showed that Neurobiotin tracer coupling (loaded by scrape loading) was reduced by an NO donor (spermine NONOate) and by Bay41-2272 (a soluble guanylyl cyclase stimulator). The effect of NO donor was reduced by the PKG inhibitor KT5823 but essentially blocked by the PKA inhibitor Rp-8-cpt-cAMPS. They also showed that PKG can phosphorylate Cx35 directly at serine 110, 276 and 289; it was already known that PKA phosphorylates Cx35 at serine 110 and 276 (Ouyang et al., 2005). Mutation of serine 289 did not abolish the effects NO donor or soluble guanylyl cyclase stimulator but mutation of serine 110 and 276 did reduce the effect of these drugs greatly. Taken together, these findings suggested that Cx35 is phosphorylated by PKG at one site (serine 289) but PKA and PKG can phosphorylate Cx35 at both serine 110 and 276. To explain the effect of both PKG and PKA on Cx35 gap junctions, Patel et al. (2006) proposed a hypothetical model whereby NO modulates Cx35 via PKG but that PKG also has an effect (cross talk) with PKA, resulting in PKA-mediated modulation of Cx35.

The nature of the cross talk between PKG and PKA was unknown, but more recently it was shown that the phosphorylation of the RIα regulatory subunit of PKA by PKG can sensitize PKA to become active even without elevated cyclic AMP (Haushalter et al., 2018). To understand this work requires a brief review of the molecular details of PKA activation. Inactive PKA consists of two regulatory and two catalytic subunits. The

general model of PKA activation posits that when cyclic AMP levels increase, the

regulatory and catalytic subunits dissociate allowing the catalytic subunit to mediate

protein phosphorylation at serine or threonine sites (Sassone-Corsi, 2012). However,

recent work has suggested that activation of the catalytic activity of PKA might proceed

without separation of the regulatory and catalytic subunits, especially at physiological

concentrations of cAMP (Byrne et al., 2016; Smith et al., 2017). There are two

regulatory subunits (RI and RII, each having α and β isoforms) and it has been known for

some time that RIα is phosphorylated by PKG (Geahlen et al., 1981; Geahlen et al.,

1982) but Haushalter et al. (2018) showed that such phosphorylation activates PKA, even

without elevation of cyclic AMP.

The work of Haushalter et al. (2018) provides one possible mechanism to explain

the results of Patel et al. (2006) showing cross talk between PKG and PKA in the

modulation of Cx35. Moreover, cross talk between PKG and PKA could explain the

effect of both PKG and PKA inhibitors on the reduction of Neurobiotin spread in goldfish

HATs. Patel et al. (2006) studied Cx35, a connexin where phosphorylation sites for both

PKA and PKG were identified. Several connexins have been associated with carp

horizontal cells, specifically Cx53.8 and Cx55.5, that are localized to both HCS and

HATs (Greb et al., 2017). However, to the best of my knowledge there has been no

published reports describing phosphorylation of these connexins. Given the effect of

both cyclic AMP and cyclic GMP on HCS gap junction coupling (Lasater and Dowling,

1985; Lasater, 1987; Lu and McMahon, 1997; Pottek et al., 1997) it seems reasonable to

propose that these connexins will be shown to have phosphorylation sites for PKA and

PKG.

Prompted by the effect of KT5823 on Neurobiotin spread related to HAT, I examined the effect of this drug on HCS receptive-field size in goldfish and Neurobiotin tracer spread in HCS of mouse retina. With respect to the former, I showed that KT5823 reduced the effect of light-adaptation on HCS receptive-field size, assessed by comparing the response to annulus and spot stimuli. With respect to the latter, I showed that bright light reduced of the spread of Neurobiotin (applied by cut loading) in calbindin-IR horizontal cells in mouse. This reduction was prevented by KT5823. I also showed, as part of these experiments, that both dopamine and nitric oxide donor reduce Neurobiotin spread in mouse HCS. Although gap junction coupling between mouse HCS has previously been shown by tracer coupling, and to be modulated by dopamine and retinoic acid (Weiler et al., 1999; He et al., 2000) the work reported here is the first to investigate the role of light conditions and NO on mouse HCS gap junction coupling. The results of the study of HCS in goldfish (receptive-field assessment) and mouse (Neurobiotin tracer coupling) are consistent with the studies of HAT in goldfish showing an effect of KT5823 on light-adaptation. With the caveat that the effect of a PKA inhibitor was not examined as part of the goldfish electrophysiology or mouse cut loading experiments, the results highlight a role of PKG in the reduction of HCS gap junction coupling and receptive-field size produced by exposure to bright light in fish and a mammal (mouse).

5.4.2 Dark-suppression

In parallel to the studies of horizontal cell light-adaptation described above, I also examined the effect of some of the same drugs on the reduction of HAT gap junction coupling during dark-suppression, the effect of prolonged darkness. I found that Neurobiotin spread after dark-suppression was reduced compared to the spread in HATs

during light-sensitization. It has been suggested previously that dark-suppression of HCS, and the corresponding reduction of HCS responsiveness (Yang et al., 1988b; Baldridge et al., 1995), is due to dopamine (Tornqvist et al., 1988; Yang et al., 1988a). Therefore, if dopamine is responsible for the reduction of Neurobiotin spread in HATs observed after 1 hr of darkness, it was expected that it would be reduced by the PKA>PKG inhibitor H89, but not the nitric oxide synthase inhibitor L-NAME or the PKG inhibitor KT5823. While L-NAME had no effect on HAT dark-suppression, it was PKG, not H89, that reduced the effect of dark-suppression on Neurobiotin spread. At face value this would suggest that PKG is involved in the mechanism of HAT dark-suppression and not NO. One possibility is that carbon monoxide (CO), shown previously to increase cyclic GMP in the retina (Cao et al., 2000), might mediate the effect of dark-suppression independent of NO. However, it was later shown that the effect of CO could be blocked by nitric oxide synthase inhibitors (Cao and Eldred, 2003) indicating that the effects of CO are mediated by NO.

In considering a possible role for PKG in dark-suppression it is worthwhile to consider the strength of the current evidence supporting the view that dopamine is the mediator of dark-suppression in HSC. In fact, the strongest evidence comes from a single report (Tornqvist et al., 1988) where both darkness and dopamine reduced Lucifer yellow dye coupling in HCS of the white perch retina. A companion paper (Yang et al., 1988a) had showed that the suppression of HCS by darkness was abolished by the D1 dopamine receptor antagonist SCH-22390 or by eliminating the endogenous source of dopamine by prior treatment with the neurotoxin 6-hydroxydopamine (6-OHDA). These data suggested that dark-suppression is mediated by dopamine. As a result, Tornqvist et al.

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(1988) concluded that the effect of darkness on HCS gap junctions must also be due to dopamine. They did not do the conclusive experiment of examining the effect of SCH-22390 or the consequence of 6-OHDA on Lucifer yellow dye coupling following darkness. It is perhaps not such an unreasonable inference to make that if dopamine affects one component of dark-suppression (HCS responsiveness) that it would not also affect the other (HCS gap junction coupling). But in light of my result, the failure of H89 to reverse the reduction of HAT Neurobiotin spread after darkness and, instead, the effectiveness of KT5823, alternate models of dark-suppression may be worthy of consideration. Given that nitric oxide synthase inhibitor did not affect HAT dark-suppression, it seems unlikely that NO is the mediator. It is difficult to propose a possible neuromodulator/pathway that would act via soluble guanylyl cyclase or PKG independent of NO. I am not aware of other activators of soluble guanylyl cyclase, other than CO (considered above), but there is evidence that PKG can be activated by other kinases, such as protein kinase C (PKC) (Hou et al., 2003). Interestingly, activation of PKC by phorbol ester has been shown to reduce HCS responsiveness and receptive-field size in turtle retina (Akopian et al., 1992) but these effects were reduced by dopamine receptor antagonists (haloperidol and fluphenazine) suggesting that, in fact, the mechanism of the phorbol ester was mediated by release of dopamine.

Obviously much more research is required to further understand the mechanism of HCS or HAT dark-suppression. A good starting point would be to investigate further dark-suppression by determining, with certainty, the effects of D1 dopamine receptor antagonists and PKA inhibitors on tracer spread through HCS and HAT gap junctions in dark-suppressed fish retina. Given the demonstrated utility of cut loading of HCS in the

mouse retina demonstrated in this thesis, this preparation could also be used to investigate if the mouse retina also shows reduced coupling of HCs gap junctions under darkness and, if so, if it is modulated by dopamine/PKA or NO/PKG pathways.

5.4.3 Flickering light

The results of my study of flickering light on HATs were the most congruent with what has been demonstrated previously in HCS. Umino et al. (1991) showed that flickering light reduced HCS receptive-field size and Lucifer yellow dye coupling, and the former was blocked by the D1>D2 dopamine receptor antagonist haloperidol. This suggested that the effect of flickering light was mediated by dopamine and this would be consistent with studies of dopamine release that found that flickering light to be a powerful stimulus for dopamine release in the retina (Weiler et al., 1997). My study of goldfish HATs demonstrated that flickering light reduced Neurobiotin spread and that this effect was blocked by the PKA>PKG inhibitor H89 but not the nitric oxide synthase inhibitor (L-NAME) or PKG inhibitor (KT5823). These results are consistent with the dopaminergic mechanism proposed for the effects of flickering light on HCS receptive-field size and gap junction coupling (see Fig. 5.1). As Umino et al. (1991) did not test the effect of a dopamine receptor antagonist on flickering light (only on receptive-field size) my data is the first to show modulation of gap junction-permeable tracer spread, in this case associated with HATs, subject to flickering light. An obvious additional future experiment would be to assess the effect of a dopamine receptor antagonist, to confirm that the results of Umino et al. (1991), from study of HCS, also apply to HATs. In addition, the effect of flickering light vs. steady light on HCS receptive-field size or gap junction coupling has not been tested on a mammalian retina. Therefore, the effect of

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flickering light on Neurobiotin spread could be examined using cut loading in the mouse retina, as shown in this thesis, and followed up with examination of the effects of a dopamine receptor antagonist and other drugs to interrogate the adenylyl cyclase/cyclic AMP/PKA pathway.

5.4.4 Limitations

5.4.4.1 Stab loading

In goldfish retina, stab loaded with Neurobiotin, it was not always possible to definitively identify loading of HATs, especially after light conditions or treatments that resulted in reduced spread from the stab site. Although the spread of Neurobiotin was clearly different from that achieved in light-sensitized retinas, in some cases it was difficult to find definitive evidence of HAT loading near the stab. As suggested in Chapter 2 (Discussion), this could indicate that light conditions or treatments reduced HAT gap junction permeability to such an extent that there was little or no Neurobiotin spread within HATs near the stab. Even in the cut loading experiments performed using mouse retina, the light condition or treatments that reduced Neurobiotin spread resulted in loading of just one or small numbers of HCS near the cut. In contrast, the effect of treatments that reduce the spread of Neurobiotin injected intracellularly into HCS results in fewer, but still numerous, coupled cells compared to control conditions (He et al., 2000; Xin and Bloomfield, 2000). But in some cases, treatments can produce a dramatic reduction in Neurobiotin spread, eventually reducing the number of labelled cells to just a single cell that was injected (Weiler et al., 1999). It may be that the conditions employed here led to reduced gap junction coupling resulting in very limited loading of HATs in the region of the stab. A way to address this issue would be to determine if treatments that

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were less potent (for example, the same drugs, but applied for shorter duration or reduced

concentrations), or a shorter period of bright light, might increase the consistency of HAT

loading within the region of the stab yet still show reduced Neurobiotin spread compared

to light-sensitized conditions.

Another complication of the stab technique was the occasional loading of structures

that were not characteristic of HATs. Based on the position, relative to HATs and

examined further by altering z-position (focus) of the microscope, this labelling was

suggested to be HCS (see Section 2.3.2). However, it is not clear that it should be

possible to visualize HCS and HATs within the same depth of focus. A possible

explanation is that the unevenness or deformation of the retinal tissue, especially in the

region near the stab, could result in portions of the distal INL being imaged at the same

focal plane as HATs. Regardless, this labelling was not a consistent feature of

Neurobiotin stab loaded retinas and did not provide means to study HCS. Perhaps the

more important question, for which I do not have an answer, is: why were HSC not

consistently labelled by the stab technique? It was expected, given their large somata and

extensive gap junction coupling, that they would have been a perfect target for stab

loading.

5.4.4.2 Protein kinase inhibitors

Another limitation of the work in the thesis is that PKG and PKA inhibitors are

not as selective as the commercial suppliers tend to suggest. The PKG inhibitor used in

this thesis, KT5283, is one of the more selective inhibitors with a K_i of 0.234 µM for

PKG and > 10 µM for PKA (Kase et al., 1987; Hidaka and Kobayashi, 1992). I used

KT5823 at 1 µM which should be relatively selective for PKG over PKA, although

perhaps it would still be more appropriate to consider KT5823 as a PKG>PKA inhibitor.

On the other hand, the PKA>PKG inhibitor H89 has a K_i of 0.05 µM for PKA and 0.5

µM for PKG (Hidaka and Kobayashi, 1992; Dostmann and Nickl, 2010). Even though

the drug was used at 0.1 µM, it is probably best to consider H89 as a PKA>PKG

inhibitor. Although this may explain the effect of both KT5823 and H89 on Neurobiotin

spread in HATs in the light-adapted goldfish retina (see Chapter 3), other results suggest

that the effect of KT5623 was not always mimicked by H89 (and vice versa). This might

be expected if there was such overlap in their effect on PKG and PKA. For example,

KT5823 had an effect (admittedly unexpected) on Neurobiotin spread in dark-suppressed

goldfish retinas but not H89. Conversely, H89 had an effect on Neurobiotin spread in

goldfish after flickering light whereas KT5823 did not. Another concern was the narrow

range over which KT5823 was effective (1 µM). Many (in fact, as far as I could tell,

most) studies of various isolated tissue preparations, where an effect of KT5823 has been

reported, typically used the drug at 1 µM (Lev-Ram et al., 1997; Michel et al., 2011;

Olivares-Gonzalez et al., 2016; Sun et al., 2020) perhaps indicating a lower limit of

effectiveness in such preparations and a concentration that is appropriate given the K_i of

KT5823. The suggestion of possible KT5823 toxicity (Dostmann and Nickl, 2010) might

explain the lack of an effect at 10 µM but perhaps indicates cause for concern even at 1

µM.

An obvious answer is to use more selective PKG and PKA inhibitors. In fact, many

of the "selective" cyclic nucleotide Rp- inhibitors are only slightly more selective than

KT5823 or H89 (Dostmann and Nickl, 2010) and some of the Rp-cAMPS inhibitors can

act as partial agonists. Lastly, the cost, in relation to the K_i, make some of them

impractical for bath application and some protein kinase inhibitors are not membrane pemeable and require intracellular injection. Nonetheless, it certainly would be worthwhile to examine the effect of other PKG and PKA inhibitors on Neurobiotin spread in goldfish HATs and mouse HCS, and receptive-field size in goldfish HCS, to verify the effects of KT5823 and H89 demonstrated in this thesis.

5.4.4.3 Off-target effects

A problem that always plagues studies in intact tissue preparations employing bath application of drugs are off-target effect. In the case of horizontal cells, a leading concern would be possible effects on photoreceptors, increasing or decreasing photoreceptor output and affecting the responses of horizontal cells but also all downstream retinal neurons. A major example of a potential confounding off-target effect in this thesis was the use of the membrane-permeable analogue of cyclic GMP. As cyclic GMP is also the key second messenger involved in phototransduction, an analogue would seem almost certain to have effects on the light responses of photoreceptors (Cobbs et al., 1985). Despite this, other studies using intact retina preparation have also employed membrane-permeable analogues of cyclic GMP (Pottek et al., 1997; Xin and Bloomfield, 2000). In both studies the cyclic GMP analogue increased the responses of HCS but the treatment also reduced HCS receptive-field size and dye or tracer coupling. The effect of cyclic GMP analogue on photoreceptors could explain the alteration of HCS responsiveness (although this was not considered by the authors of these studies) but the effects on receptive-field size and dye or tracer coupling were considered to represent the direct effect of cyclic GMP analogue on HCS, not a result of an effect on photoreceptors

5.5 Implications

5.5.1 Triphasic adaptation in the mammalian retina

As described in Chapter 4, Neurobiotin tracer coupling of HCS in mouse has been shown to be reduced by dopamine (He et al., 2000) and retinoic acid (Weiler et al., 1999) and in this thesis I show that NO also reduces HCS tracer coupling in mouse. An effect of NO on HCS was shown previously in rabbit retina, where both NO donor and membrane-permeable cyclic GMP reduced receptive-field size and Neurobiotin tracer coupling (Xin and Bloomfield, 2000). No prior study in mouse has demonstrated light condition-dependent changes in HCS receptive-field size or gap junction coupling but (Xin and Bloomfield, 1999) discovered evidence of "triphasic adaptation" in rabbit retina HCS. In dark-adapted retina the gap junction coupling between rabbit HCS was weak. Exposure to dim light increased coupling but exposure to bright light reduced coupling. There has been no subsequent study (in rabbit retina) that determined if the effect of darkness or bright light are affected by a nitric oxide synthase inhibitor, such as L-NAME. Nonetheless, this result indicated that triphasic adaptation of HCS is not unique to the fish retina. In this thesis I add to this evidence by showing the reduction of Neurobiotin spread in mouse HCS when retinas were exposed to bright light, compared to retinas maintained under dim light conditions. It remains to be determined what, if any, effect darkness has on HCS gap junction coupling in mouse retina.

Another mammalian retinal neuron in which the modulation of gap junction coupling has been studied in some detail is the coupling between AII amacrine cell. AII amacrine cells are part of the rod pathway and carry signals from rod bipolar cells to ON cone bipolar cells via gap junctions and to OFF cone bipolar cells via glycinergic

synapses (Kolb and Famiglietti, 1974). In addition, AII amacrine cells are coupled to each other by Neurobiotin-permeable gap junctions (Vaney, 1991). This coupling was shown to be reduced by dopamine (Hampson et al., 1992). A similar result was reported Xia and Mills (2004) who also found that the coupling between AII amacrine cells and ON cone bipolar cells was preferentially reduced by NO. The coupling between AII amacrine cells was also found to be modulated by light condition and followed the "triphasic" pattern (Bloomfield et al., 1997). In the dark, the coupling between AII amacrine cells was reduced compared to the coupling under dim light. When exposed to bright light, coupling decreased.

The coupling between mammalian HCS and AII amacrine cells was highlighted because of the evidence that these neurons show, like fish horizontal cells, evidence of triphasic adaptation. Of course, many other types of retinal neurons are coupled by gap junctions and affected by neuromodulators (Völgyi et al., 2013) but less is known about the conditions that would promote the endogenous action of neuromodulators on these gap junctions.

5.5.2 Significance to the generation of center-surround receptive-fields

Horizontal cells are thought to be key contributors to the receptive-field surround of bipolar cells and this is reflected in the receptive-field properties of ganglion cells (Naka and Nye, 1971; Naka and Witkovsky, 1972; Marchiafava, 1978; Toyoda and Tonosaki, 1978a; Toyoda and Tonosaki, 1978b; Toyoda and Kujiraoka, 1982; Mangel and Dowling, 1987; Mangel, 1991). Therefore, the reduction of HCS gap junction coupling during dark-suppression (Tornqvist et al., 1988) could explain why the receptive-field surround of bipolar cells and ganglion cells are reduced during darkness (Barlow et al., 1957;

Rodieck and Stone, 1965; Maffei et al., 1971; Yoon, 1972; Enroth-Cugell and Lennie, 1975; Barlow and Levick, 1976; Peichl and Wässle, 1983). The reduction of the surround would increase the sensitivity of ganglion cells to light by removing the inhibitory influence of the surround but with a loss of contrast detection.

What would be the utility of reducing HCS receptive-field size after exposure to bright light? One could predict that the receptive-field surround would be reduced in size but without a loss of strength. This would mean that the area of the visual field sampled by the surround would be smaller and, therefore, might permit spatial contrast detection to be done more locally. It is also worth considering that teleost horizontal cell light-adaptation is produced by either steady illumination or flickering light, although the mechanisms appear to be different (NO in the case of the former, dopamine in the case of the latter). It is not clear why these different processes exist, but perhaps it allows horizontal cell receptive-field size to be decreased as ambient illumination level rises in either spatial contrast-rich (producing flickering illumination) or spatial contrast-poor (steady illumination) natural scenes.

The receptive-field organization of ganglion cells with respect to ambient luminance is probaby much more complex than previously imagined. Tikidji-Hamburyan et al. (2015) showed that mouse and pig ganglion cell responses exhibited specific changes at each of seven ambient light levels covering the scotopic to photopic, including the appearance and disappearance of ON responses in OFF cells and vice versa. Differences in the contribution of stimuli that impinged on the periphery, activating the surround, were also altered over the seven ambient light levels, but no consistent pattern with respect to light level was reported. Indeed, distinct effects of peripheral stimulation

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were observed at all light levels, from scotopic to photopic, suggesting that some ganglion cells possess a receptive field surround even under scotopic conditions.

Descriptions of horizontal cell contributions to center-surround receptive-field organization depend on feedback or feedforward connections made by HCS with photoreceptor terminals (feedback) or bipolar cell dendrites (feedforward), respectively (Murakami et al., 1982; Fahrenfort et al., 2005; Thoreson and Mangel, 2012). However, the HATs in the teleost retina, studied in this thesis (Chapters 2 and 3) do not make contacts with either photoreceptor terminals or bipolar cell dendrites and instead make synaptic contacts with amacrine cells or bipolar cells in the INL (Dowling et al., 1966; Marc and Liu, 1984; Marshak and Dowling, 1987). The function of these contacts has never been fully established and it is not known what neurotransmitter would mediate synaptic transmission at these sites. However, in goldfish retina, ~60% of the contacts from HATs were onto bipolar cells, leading to the suggestion that such contacts could contribute to the generation of the inhibitory surround in bipolar cells (Marshak and Dowling, 1987). However, in catfish, current injection into HATs produced responses only in amacrine cells (Sakai and Naka, 1985). This result is surprising, given the magnitude of extent of HAT-bipolar cell contacts identified in goldfish. It is possible this reflects a species difference. In fact, Sakai and Naka (1986) were unable to identify HAT-bipolar cell synaptic contacts in catfish retina. If it is indeed the case that goldfish HATs represent another pathway for the surround to be contributed to bipolar cells, it would also make sense that changes in gap junction coupling between HATs would be modulated in parallel with changes in gap junction coupling between HCs so that the spatial extent of the surround input was similar at both levels.

As discussed above, another role of HAT gap junction coupling may be to shorten the effective length of the axon allowing electrotonic current flow from HCS to HATs (Yagi, 1986) thereby allowing for the signal from HCS to be passed to HATs and affect bipolar cells and amacrine cells. This might suggest that under condtions where HAT gap junctions are uncoupled, signals might no longer pass from HCS to HATs and vice versa. Although such an effect was not seen by Shigematsu and Yamada (1988) (i.e. dopamine did not affect HAT receptive-field size and certainly did not markedly reduce the amplitude of light responses in HATs) it would be interesting to examine this issue furthe, including examing the effect of NO.

It is also worth considering further the reported synaptic contacts of HATs onto the processes of dopaminergic interplexiform cells (DAergic IPCs) (Marshak and Dowling, 1987). This suggests that HATs could be involved in the control of dopamine release in the retina that, in turn, modulates HCS and HAT gap junction coupling. The neurochemical identity of the synaptic contacts made by HATs is not known with certainty. Horizontal cells (both HCS and HATs) are thought to contain and release GABA (Marc et al., 1978; Ayoub and Lam, 1984) but this has only been demonstrated definitively for the H1 horizontal cells. Nonetheless, if the neurotransmitter used by HATs is inhibitory (e.g. GABAergic) to DAergic IPCs (Negishi et al., 1983), and if released during depolarization (Schwartz, 1982), HATs would exert an inhibitory influence on DAergic IPCs during low light conditions (when horizontal cells are depolarized) and this inhibition would decrease with elevated light levels (when horizontal cells hyperpolarize). This is not consistent with the observations that dopamine is not released by bright steady light (light-adaptation) and released during

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darkness (dark-suppression). Therefore, the mechanism by which HATs influence

DAergic IPCs is likely to be more complex than the simple model described above.

5.6 Conclusions and Key Future Directions

5.6.1 Conclusions

Within the limitations described above and throughout the thesis, the conclusions of

the thesis work reported here are:

1) HAT gap junction coupling in the goldfish retina can be revealed using stab
loading of Neurobiotin.

2) HAT gap junction coupling in the goldfish retina is reduced by dopamine, nitric
oxide, all-trans retinoic acid as well as by cyclic GMP and cyclic AMP analogues.

3) Gap junction coupling of goldfish HATs show triphasic adaptation to different
levels of ambient illumination, with a decrease of gap junction coupling after darkness
(dark-suppression) or bright light exposure (light-adaptation) relative to conditions where
retinas were exposed only to dim light (light-sensitization).

4) The effect of light-adaptation on goldfish HAT gap junction coupling involves
NO, mediated by cyclic GMP and PKG. A possible role for PKA was also identified.

5) The effect of light-adaptation on goldfish HCS receptive-field size involves
PKG.

6) Mouse HCS gap junction coupling can be revealed using cut loading of
Neurobiotin.

7) Mouse HCS gap junction coupling is reduced by NO, dopamine and bright light
and the effect of bright light involves PKG.

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8) The effect of darkness (dark-suppression) on goldfish HAT gap junction coupling involves PKG but not NO or PKA.

9) The effect of flickering light on goldfish HAT gap junction coupling involves PKA but not NO or PKG.

Taken together, conclusions 2-5 and 7 support the guiding hypothesis of the thesis (Section 1.13) that NO, via the activity of PKG, is the mechanism by which horizontal cell gap junctions are uncoupled following exposure to periods of bright, sustained illumination (light-adaptation).

5.6.2 Future Directions

A key future direction would be to study the phosphorylation of the gap junction proteins (connexins) mediating the coupling of goldfish (*Carassius auratus*) HCS and HATs (e.g. Cx53.8 or 55.5) and mouse HCS (Cx57). The phosphorylation of these Cxs could be studied by cloning of the Cx genes and examination in expression systems, as was done for Cx35 by Patel et al. (2006). This would be very useful, especially to determine the phosphorylation sites and determine the relative contributions of PKG and PKA to Cx53.8 or Cx55.5 phosphorylation. However, this would not allow for investigation of the phosphorylation achieved *in situ*, where the Cxs would be subject to endogenous conditions, but could still be manipulated by exogenous treatments. One way to approach this would be to use an antibody that can label the phosphorylated Cx in Western blots. Changing phosphorylation level can alter the molecular weight of the Cx. Therefore, homogenates from retina subject to different illumination conditions or drug treatments could be examined to determine the extent of phosphorylation of the gap junction protein under different light condition and drug treatments.

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Clearly additional research is required to understand the mechanism of dark-suppression. The challenge of dark-suppression is that the condition is very sensitive to light, meaning that standard approaches to record HCS responses quickly shift retinas from dark-suppression to light-sensitized (Baldridge et al., 1995). This is where bulk loading of Neurobiotin can be especially valuable, as all the manipulations can be done in total darkness using an infrared imager. Therefore, another key future direction could be to use stab (goldfish) or cut (mouse) loading to study the mechanism by which HCS and HAT gap junction coupling is reduced following darkness. This would include comparing the effect of dopamine receptor antagonist vs. nitric oxide synthase inhibitor to determine the contribution of dopamine and/or NO to dark-suppression.
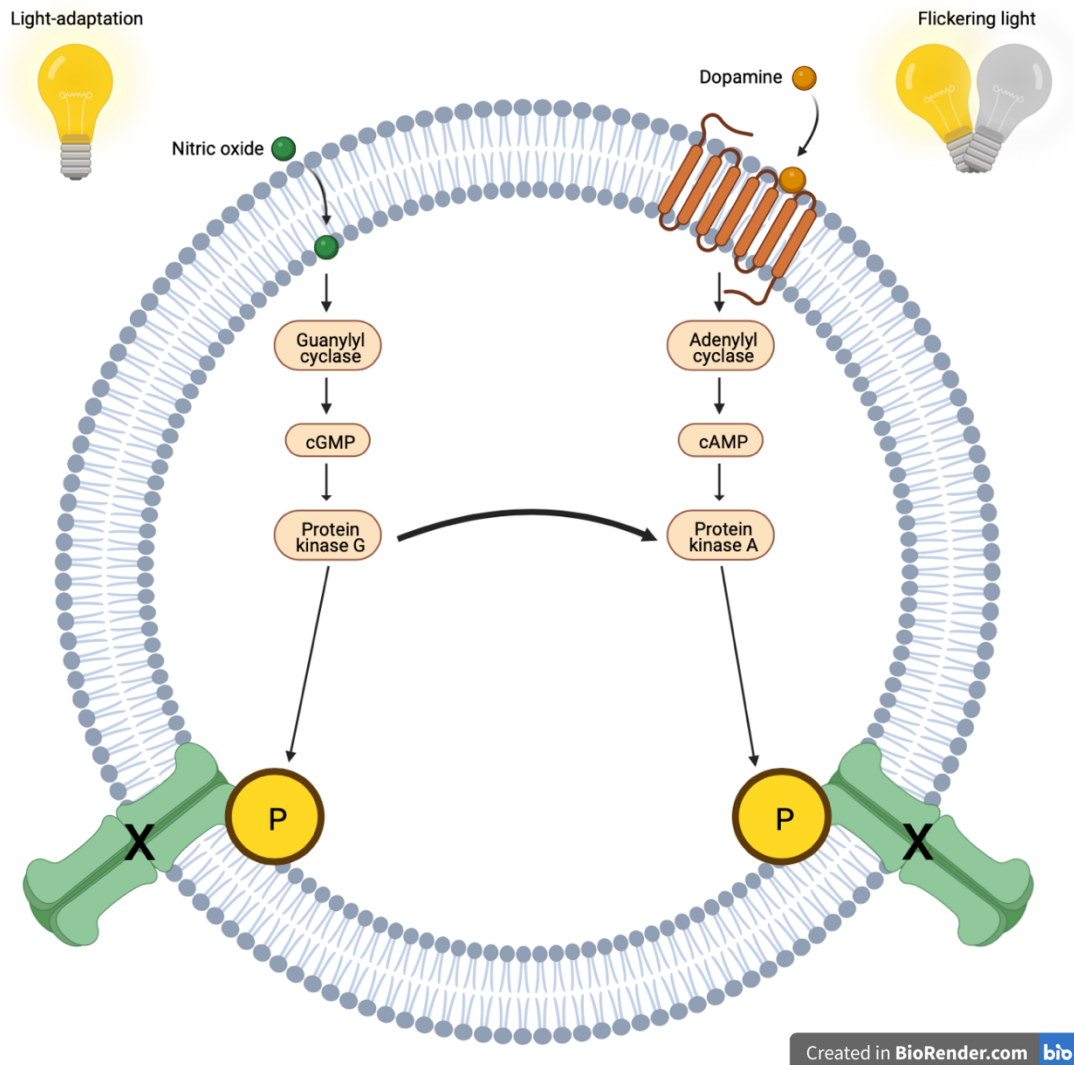
Figure 5.1. Summary diagram of possible mechanism of light-adaptation and flickering light on horizontal cell gap junction coupling.

Light-adaptation (produced by bright steady light) and flickering light have both been shown to decrease horizontal cell gap junction coupling. Findings in this thesis, and from previous studies, suggest that the mechanism of light-adaptation involves NO that increases the activity of soluble guanylyl cyclase, thereby increasing levels of cyclic GMP, leading to activation of PKG. In the case of flickering light, dopamine binds to D1 dopamine receptors, increasing adenylyl cyclase activity and the level of cAMP, leading to activation of PKA. Both PKG and PKA have been shown to phosphorylate connexins resulting in increased gap junction resistance (Lampe and Lau, 2000; Patel et al., 2006). The reversal of such phosphorylation would likely be mediated by phosphatase enzymes (Lochner and Moolman, 2006). A possible interaction between PKG and PKA, that leads to PKA activation, has been suggested (Patel et al., 2006; Haushalter et al., 2018) and could explain the effect of PKA inhibitor on HAT light-adaptation described in this thesis. Created with BioRender.com

APPENDIX A:
IMMUNOREACTIVITY FOR MELANOPSIN IN GOLDFISH AND ZEBRAFISH IS FOUND IN DOPAMINERGIC INTERPLEXIFORM CELLS

A.1 Introduction

The melanopsin photopigment, well studied in mammals, has both image-forming and non-image-forming functions such as contrast detection (Mouland et al., 2017; Sonoda et al., 2018) and signalling ambient light level to the hypothalamus for circadian rhythm entrainment and the pupillary light reflex (Ruby et al., 2002; Markwell et al., 2010), respectively. Though much of the focus in the current literature is on mammals, melanopsin was first isolated in a lower vertebrate, *Xenopus* (Provencio et al., 1998).

Seminal work in another non-mammalian vertebrate, the zebrafish, found labelling for melanopsin proteins ubiquitously throughout the retina, in all major classes of retinal neuron (Davies et al., 2011). This phenotype is unusual when compared to the characteristic restriction of melanopsin to a small subset of intrinsically photosensitive retinal ganglion cells (ipRGCs) in the mammalian retina (Provencio et al., 2000). Though seemingly over-abundant, endogenous melanopsin present in retinal horizontal cells has been proven to be functional in a few teleost species including roach (Jenkins et al., 2003) and catfish and goldfish (Cheng et al., 2009). In horizontal cells, as well as in other retinal interneurons, the presence of teleost melanopsin implies that it could affect visual processing.

Dopaminergic neurons are of special interest with relation to melanopsin as the sensitivity of melanopsin to ambient light level have been linked to dopamine-dependent processes in the mouse retina (Zhang et al., 2012; Prigge et al., 2016). Light-adaptation

relies on dopamine signalling to allow photoreceptors to adapt the retina to brighter light and avoid saturation. Melanopsin's involvement in light-adaptation in the murine retina would indirectly promote this processes in the light by potentially driving dopamine release through retrograde ipRGC signalling. The teleost retina presents a special case where the ubiquity of melanopsin suggests the possibility that dopaminergic interplexiform cells (DICs), the resident dopaminergic neurons of the teleost retina, may themselves express melanopsin and be intrinsically photosensitive. This would allow teleost retinas faster, more direct control over dopamine release and its sequelae.

Here, we present the labelling of three melanopsin antibodies and investigate whether the presence of melanopsin can be detected in goldfish and zebrafish dopaminergic interplexiform cells.

A.2 Methods

A.2.1 Animals

Male and female goldfish and zebrafish were housed on a 12-hour light/dark schedule. Fish were euthanized using a solution of 300 mg of MS-222/L of tank water buffered 2:1 by sodium bicarbonate (Westerfield, 1993; Matthews and Varga, 2012).

For wholemounts, fish were dark-adapted for 1 hour to allow for retinomotor movements to displace the retina from the choroid and RPE and eyes were enucleated. The optic nerve was cut to isolate the retina and the retina was placed in 200 mM sucrose solution in phosphate buffer (PB) for 15 minutes at room temperature before 1 hour of fixation with a 2% paraformaldehyde (PFA) solution containing 134 mM HEPES and 200 mM sucrose solution at room temperature.

For sections, goldfish eyes were enucleated and the anterior portion of the eyes cut into eyecups. Eyecups were placed in the 200 mM sucrose in PB solution for 15 minutes at room temperature before being placed in the 2% PFA fixative solution overnight at 4°C. Zebrafish heads were cut and the same fixation protocol was used. Fixed zebrafish heads and goldfish eyecups were cryoprotected in 30% sucrose overnight at 4°C. Frozen tissue sections were made at 18 – 20 μm thicknesses using a cryostat.

A.2.2 Immunohistochemistry

Immunolabelling for melanopsin was conducted using one of two melanopsin antibodies. An antibody, pas350 (1:100), raised against a 13-amino acid synthetic peptide sequence (CVPFPTVDVPDHA) for a conserved region of three melanopsin proteins was used to label opn4m-1, 2, and 3 (Davies et al., 2011). Alternatively, opn4a, a combination of monoclonal antibodies raised against synthetic peptides for the N-terminus region (MMSGAAHSVRKG-TRIVESLSAWND-NDSVMSAYRLVD) of opn4m-1 (1:400; F1QGX5, Abmart). Finally, an antibody, opn4l, was used to label opn4m-2 (1:200; MyBioSource).

To label for retinal neuron subtypes, labelling was conducted with antibodies for tyrosine hydroxylase (TH) anti-mouse (1:500; IHCR1005-6, Millipore Sigma), TH anti-rabbit (1:500; AB152, Millipore Sigma), γ-aminobutyric acid (GABA) anti-rabbit (1:400; A2052, Millipore Sigma), or choline acetyltransferase (ChAT) anti-goat (1:250; AB144P, Millipore Sigma). Above labelling concentrations are for sections while for wholemounts, all primary antibodies were used at 1:100. Appropriate secondaries were used for fluorescence and all images were acquired using a Nikon D-Eclipse C1 laser scanning confocal microscope.

A.2.3 Neurobiotin stab

As modified from previous cut loading experiments (Choi et al., 2012), dark-adapted flat mounted retinas were dissected in 5% CO_2 and 95% oxygenated Ringer's solution containing (mM): 130 NaCl, 20 $NaHCO_3$, 2.5 KCl, 10 glucose, 1 $MgCl_2$, and $CaCl_2$ at pH 7.4. Retinas were injected 4 to 5 times with approximately 20 µL total 5% neurobiotin tracer (MJS BioLynx Inc.) dissolved in Ringer's solution using a Hamilton syringe and needle (Hamilton Company). Retinas were incubated for 15 minutes in the dark before fixation. Retinas were subsequently washed in PB overnight at 4°C. Fluorescent labelling of the neurobiotin tracer was conducted with streptavidin (Life Technologies) used at 10 µg/mL. Co-labelling with melanopsin was conducted as previously described.

A.2.4 Western blot

Western blots were conducted as slightly modified from experiments on tiger salamanders (Sherry et al., 2001). Retinas were isolated and homogenized in 25 mM Tris buffer (pH 7.0) on ice. Retinal membranes were pelleted by centrifuging at 11 8000 g for 20 minutes at 4°C. This formed a crude pellet which was vortexed and a supernatant, both of which were analyzed for protein concentration with a Bradford assay. 1 µg/µL of protein was solubilized at 95°C for 5 minutes and 10 µL of protein was loaded per lane of a 10% acrylamide gel. Transferred blot membranes were blocked in 5% dried milk and labelled using opn4a (1:250) and opn4l (1:2000) and actin (1:50 000) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000) as controls.

A.3 Results

A.3.1 pas350 labels neurons throughout the inner nuclear and ganglion cell layers

Previously in zebrafish, the pas350 antibody labelled retinal neurons in the outer nuclear layer (ONL), INL, as well as the (GCL; (Davies et al., 2011). Here, we focussed on the INL and GCL of goldfish and zebrafish where labelling of presumptive horizontal, bipolar, amacrine, and ganglion cells was observed (Fig. A.1). Labelling of pas350 tended to be present at the circumference of cells and not near the nucleus, presumably labelling melanopsin proteins at the cell membrane (Fig. A.1A). Some subtypes of retinal neuron which were pas350-positive could be presumed by their morphology or location within the retina. Presumptive amacrine cells (Fig. A.1B), bipolar cells (Fig. A.1C), and ganglion cells (Fig. A.1D) were often seen labelled by the pas350 antibody.

Punctate pas350 labelling was observed at the OPL and IPL. At the OPL, this labelling was consistently present near presumptive cone pedicles.

A.3.2 opn4a labels neurons throughout the inner nuclear and ganglion cell layers

A commercially available opn4m-1 antibody, opn4a (Abmart) showed a similar pattern of labelling as pas350 (Fig. A.2A, B). Cell bodies in the INL and GCL of zebrafish and goldfish retinas were positive for opn4a. Punctate labelling was also present at the outer and IPL. Pre-absorption of opn4a using a synthetic peptide resulted in the abolishment of any relatively robust labelling in both the zebrafish and goldfish retinas (Fig. A.2C-F).

A.3.3 opn4l labels zebrafish retinal neurons in the inner nuclear layer

An antibody purportedly specific for the opn4m-2 protein, known here as opn4l showed sparse labelling in the proximal INL of a small population of presumptive amacrine cells. In addition to this labelling, immunofluorescence at the level of horizontal cells was abundant in both presumptive cell bodies as well as their axon terminals (Fig. A.3). Labelling was not observed in the goldfish retina.

A.3.4 A western blot labels multiple bands of protein in goldfish and zebrafish

A western blot was conducted using the opn4a as well as the opn4l antibodies (Fig. A.4). Labelling with opn4a revealed at least one band of approximately 55 kDa mass with goldfish retinal protein and multiple bands in zebrafish protein. The opn4l antibody produced labelling only against zebrafish protein in at least three bands from 50 to 70 kDa mass.

A.3.5 Dopaminergic retinal neurons are melanopsin-immunoreactive

Sections labelled for pas350, opn4a, and opn4l were also stained for TH as a marker of presumptive dopaminergic interplexiform cells (DICs). In the goldfish, dopaminergic cells were co-labelled with pas350 at a rate of 81.0% (n = 42; Fig. A.5A-C) and opn4a at a rate of 95.7% (n = 47; Fig. A.6A-C). In the zebrafish, 39.1% of dopaminergic cells were co-labelled with pas350 (n = 46; Fig. A.5D-F) and 68.1% of dopaminergic cells were co-labelled with opn4a (n = 47; Fig. A.6D-F). Co-labelling of TH and opn4l was found at almost every instance of a cell body labelled in the proximal portion of the INL (Fig. 7).

Melanopsin-positive cells as well as DICs, if not double-labelled by both a melanopsin antibody and TH, were occasionally found with the puncta of the opposite cell type apposed to their somas and or processes (Fig. A.5G-I).

A.3.6 HATs are melanopsin-immunoreactive

HCS were often faintly labelled by both pas350 and opn4a. In goldfish, labelling of H1-type HATs was widespread. When retinas were injected with neurobiotin, a cell tracer that diffuses through gap junctions, HATs were more easily distinguished and almost always double-labelled by pas350 (Fig. A.8E). Labelling with opn4l (Fig. A.3) also showed was similar but denser at the level of presumptive horizontal cells and their axon terminals in zebrafish.

A.4 Discussion

Melanopsin immunoreactivity as determined by labelling with the pas350 and opn4a antibodies exhibits labelling throughout both the goldfish and zebrafish retinas. Previously, it was observed that the zebrafish retina exhibits ubiquitous presence of melanopsin in photoreceptors, interneurons, and ganglion cells (Davies et al., 2011). To our knowledge, immunolabelling for melanopsin has not yet been observed in the goldfish retina. Though the ubiquity in both goldfish and zebrafish suggests many possibilities for function, the possibility of redundancy is not impossible as corneal melanopsin in rodents shows no as yet discovered functional responses (Delwig et al., 2018).

Goldfish melanopsin expression in horizontal cells has been previously predicted from functional experiments (Cheng et al., 2009). Here, melanopsin immunofluorescence was observed in presumptive goldfish and zebrafish horizontal cells and at goldfish and

zebrafish HATs. HATs form an extensive network in the goldfish retina while making synapses with a variety of amacrine, interplexiform, and bipolar cells. As yet, the function of goldfish HATs and unique displacement deep into the INL is not yet completely understood (Marc and Liu, 1984; Yazulla et al., 1984; Sakai and Naka, 1986; Marshak and Dowling, 1987; Kamermans et al., 1990). Less is known about the presence of HATs which descend into the INL in mammals, but even humans have been cited as having processes in the INL as opposed to solely the OPL. In general, human horizontal cells share many physiological characteristics with those of their fish counterparts (Picaud et al., 1998).

The observation that goldfish and zebrafish HATs are melanopsin-immunoreactive suggests a capacity for intrinsic photosensitivity which may contribute to horizontal cell feedback or to horizontal cell network coupling. Although zebrafish HATs have much finer processes than those of goldfish which present challenges in imaging their labelling was sufficiently visualized here. Alternatively, although axon-bearing zebrafish horizontal cells may express melanopsin in their terminals, they may function differently than goldfish HATs due to their thinner morphology and more distal termination in the retina (Connaughton et al., 2004).

Melanopsin immunoreactivity was hypothesized in goldfish and zebrafish DICs as a consequence of the sheer presence of previously described retinal melanopsin in zebrafish (Davies et al., 2011). Many TH-immunoreactive cells were also melanopsin-immunoreactive in both the goldfish and zebrafish as determined by three different melanopsin antibodies. This finding suggests a direct path for melanopsin to drive dopamine release, influencing horizontal cell coupling and contributing to light-

adaptation by shifting the response sensitivity of photoreceptors in the fish retina,

processes influenced by dopamine (Green et al., 1975; Baldridge et al., 1993). This is

analogous to the recent observation in the mouse that ipRGCs send retrograde axo-axonal

contacts to dopaminergic amacrine cells, influencing dopaminergic processes in the retina

and contributing to light-adaptation (Zhang et al., 2012; Prigge et al., 2016). More

recently, no detectable change in retinal dopamine was observed when melanopsin

stimuli were driven in the mouse, indicating that if relevant processes are engaged by

melanopsin-specific activity, it may be in an indirect way (Perez-Fernandez et al., 2019).

Ultimately, the present finding in fish does not exclude the possibility that just as in mice,

ipRGCs or maybe even other melanopsin interneurons in the retina may contact DICs to

influence dopamine release in the light. Cellular tracing and genetic labelling for synaptic

markers may help to determine this possibility, especially in the zebrafish where

deducing synaptic contacts in the retina may be more challenging.

In many fish, as in goldfish and zebrafish, retinomotor movements alternately

contract and elongate rods and cones in the dark and light (Burnside and Nagle, 1983b;

Menger et al., 2005). This circadian repositioning of photoreceptors which optimizes

visualization is in part controlled by dopamine, which, in the fish retina, is found in DICs

(Dearry and Burnside, 1988). This gives melanopsin another putative purpose for being

present in goldfish and zebrafish DICs.

As for the wider presence of melanopsin outside of DICs and horizontal cells,

melanopsin's contributions there may also mediate retinal circuitry and vision. Network-

adaptation provides adjustment to the sensitivity of the retinal circuitry of interneurons

beyond the cone-dominated, bright light process of light-adaptation. Melanopsin found in

teleost retinal interneurons, through depolarizations, could contribute to this process of network-adaptation, driving the potential of neurons into actionable parameters where they are more likely to respond to environmental stimuli. Network-adaptation operates in both low and bright light intensities (Green et al., 1975). Though melanopsin-elicited responses in the teleost have been so far mostly tested indirectly and with photopic stimuli (Jenkins et al., 2003; Cheng et al., 2009), lower light stimuli have been effective in eliciting powerful responses in mouse ipRGCs (Sonoda et al., 2018), in line with the wide breadth of luminosity over which network adaptation functions (Green et al., 1975). Additionally, like the effects of network-adaptation (Vaquero et al., 2001), melanopsin-mediated responses are expected to last for extended periods of time with putative melanopsin responses from the goldfish lasting for several minutes, beyond the scope of recording sessions (Jenkins et al., 2003; Cheng et al., 2009).

Further investigation into the functionality of melanopsin-elicited responses will elucidate more the effects on retinal adaptation in the fish, adding to our understanding of melanopsin's role in intra-retinal image-formation.
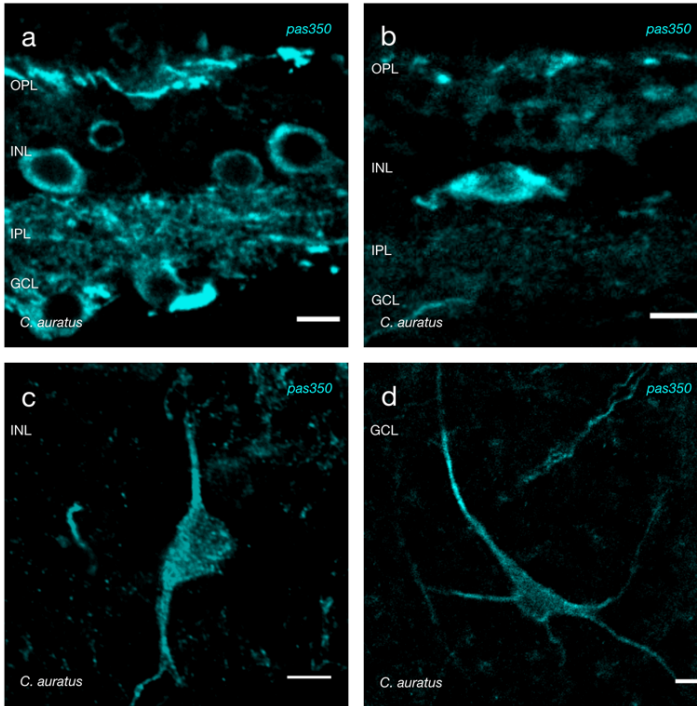
Figure A.1. Goldfish section and wholemount pas350 immunofluorescence is found across the inner retina

A) Many cell bodies in the INL of presumptive horizontal, bipolar, and amacrine cells are pas350-positive. pas350-positive presumptive amacrine and ganglion cells were observed in the GCL. Punctate labelling is present at the OPL and IPL. B) A presumptive amacrine cell in the proximal portion of the IPL is observed. C) A compressed z-stack of a presumptive bipolar cell in the INL is observed. D) A compressed z-stack of a presumptive ganglion cell in the GCL is observed. Bars = 10 μm.
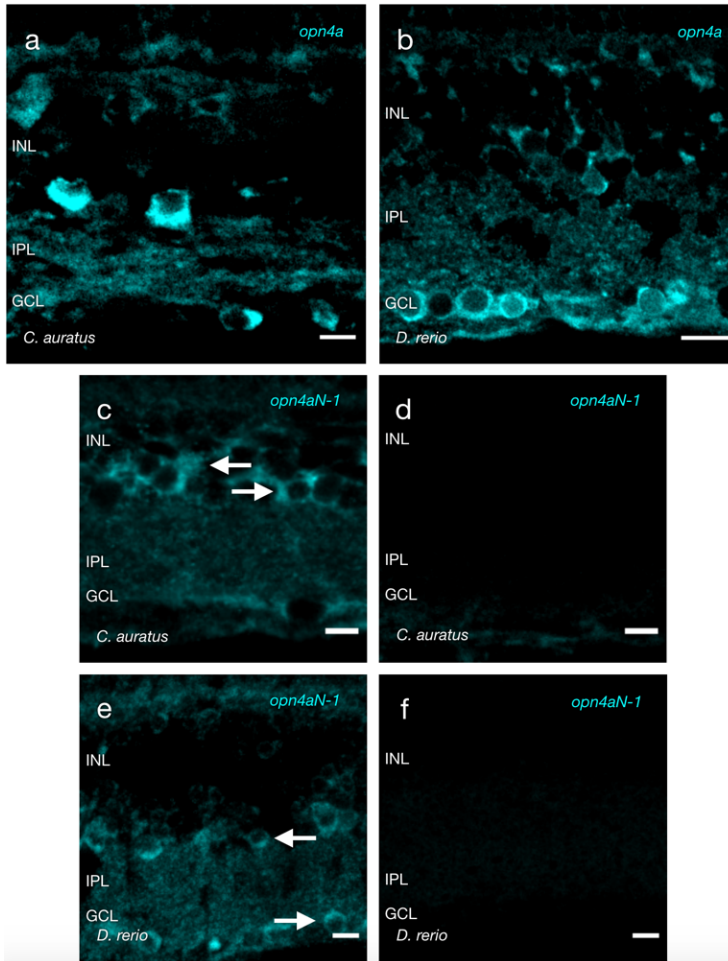
Figure A.2. Goldfish and zebrafish opn4a immunofluorescence is ubiquitous through the inner retina.

A) A collapsed z-stack shows immunofluorescence for opn4a in the inner nuclear and GCL of presumptive goldfish retinal interneurons and ganglion cells respectively. B) Zebrafish opn4a immunofluorescence shows signal in the INL at the level of presumptive horizontal, amacrine and bipolar cells, as well as cells in the GCL. Punctate labelling throughout the IPL is also visible. C-F) Peptide pre-absorption of opn4aN-1, a single monoclonal antibody from the opn4a cocktail, eliminated labelling in both goldfish and zebrafish retinas. Bars = 10 μm.
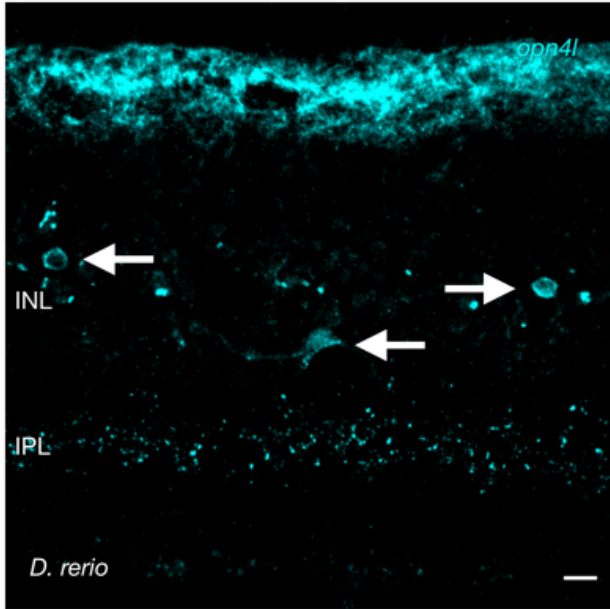
Figure A.3. opn4l labels a subset of cells in the zebrafish retinal INL.

Presumptive zebrafish horizontal cells and their thin axon terminals, which form a plexus, exhibit labelling at the distal INL (top of the image). Arrows point to opn4l-positive cell bodies in the proximal INL. Punctate labelling is present in the IPL. Bar = 10 μm.
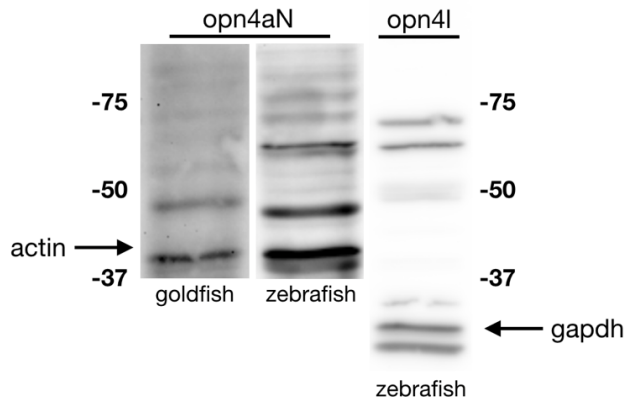
Figure A.4. Goldfish and zebrafish retina western blots using opn4a and opn4l antibodies reveals multiple bands.

A western blot carried out using goldfish and zebrafish retinal protein revealed multiple bands of protein for both opn4a as well as opn4l. opn4l did not result in any labelling in the goldfish.
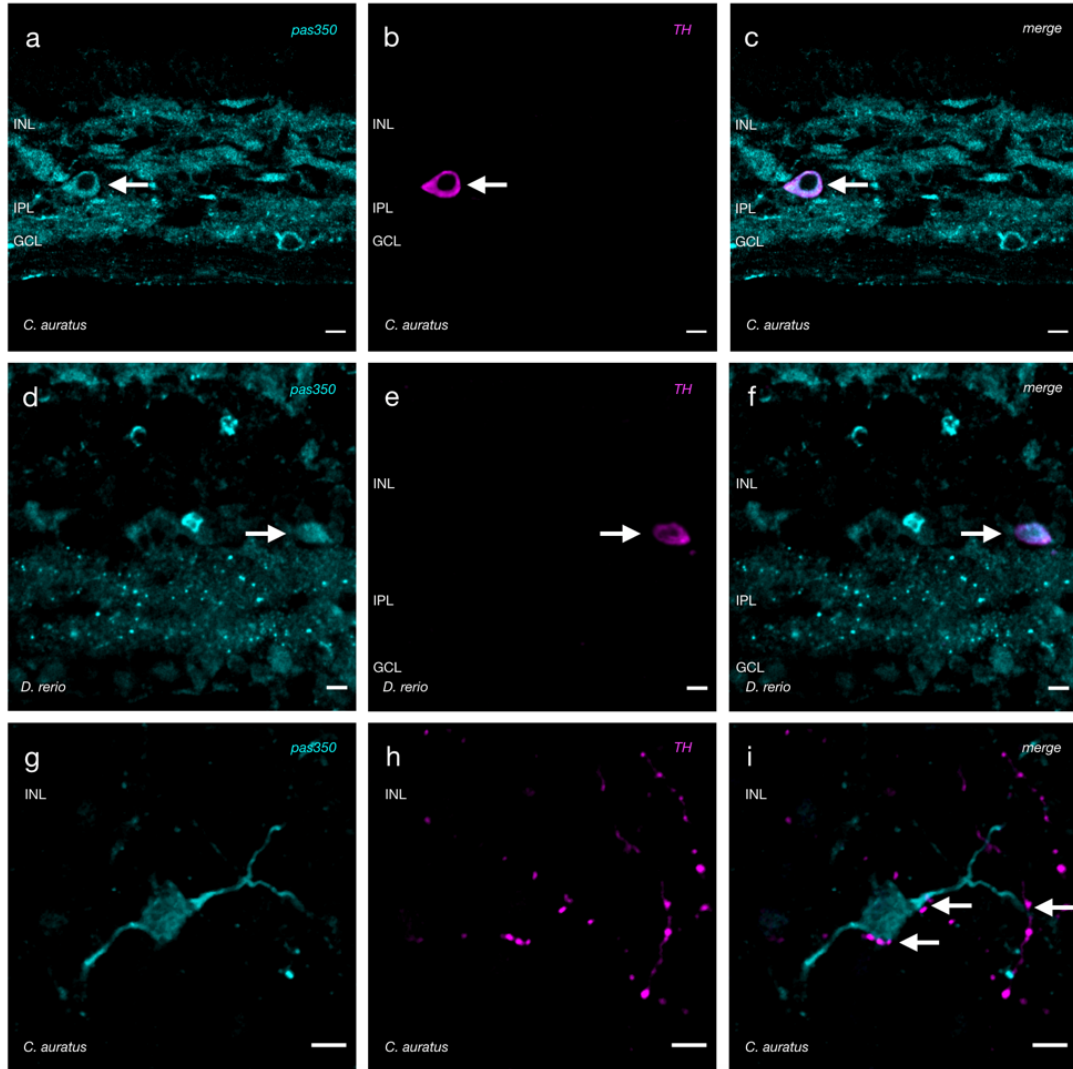
Figure A.5. TH and pas350 co-label in the goldfish and zebrafish retinas.

A-C) Co-labelling of pas350 and TH is found in the INL (arrows) in goldfish as well as in D-F) zebrafish (arrows). G-I) A pas350-labelled cell has TH puncta apposed its soma and processes (arrows). Bars = 10 μm.
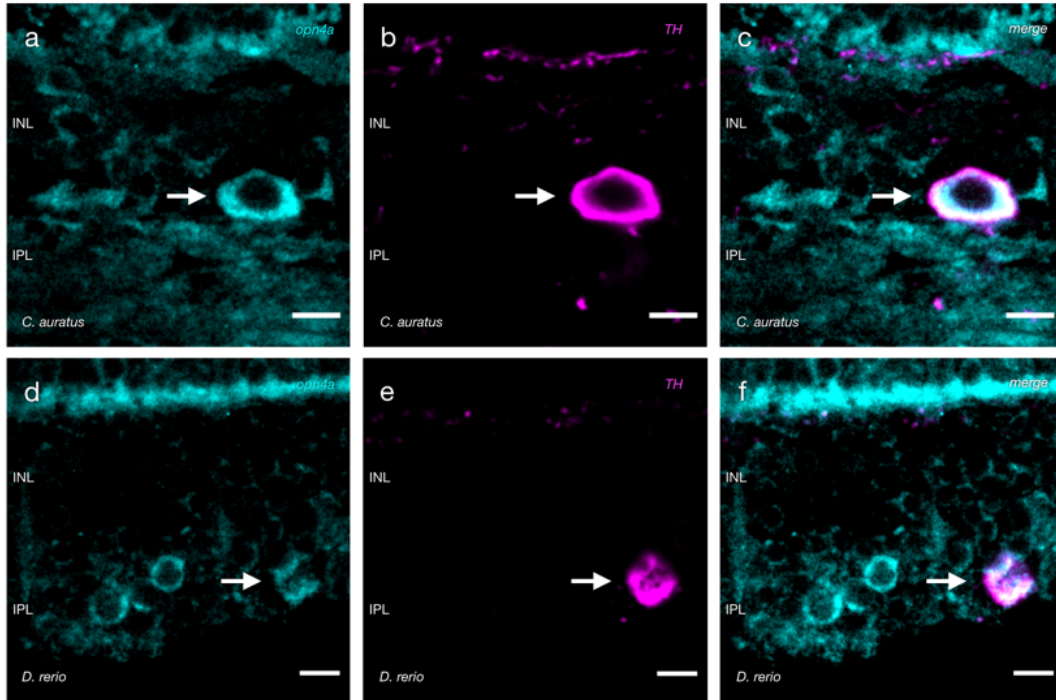
Figure A.6. TH and opn4a co-label in the goldfish and zebrafish retinas.

A-C) Co-labelling of opn4a and TH is found in the INL (arrows) in goldfish as well as in D-F) zebrafish (arrows). G-I) A pas350-labelled cell has TH puncta apposed its soma and processes. Bars = 10 μm.
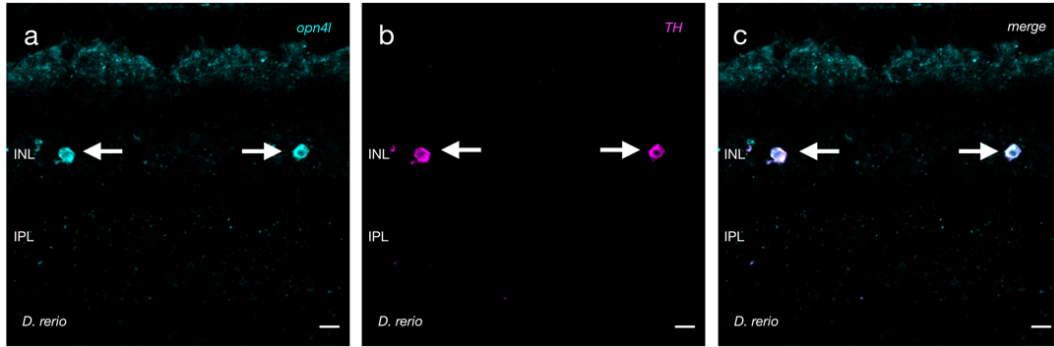
Figure A.7. TH and opn4l co-label in the zebrafish retina.

A-C) Zebrafish labelling for opn4l and TH shows co-labelling in the INL. Bars = 10 µm.
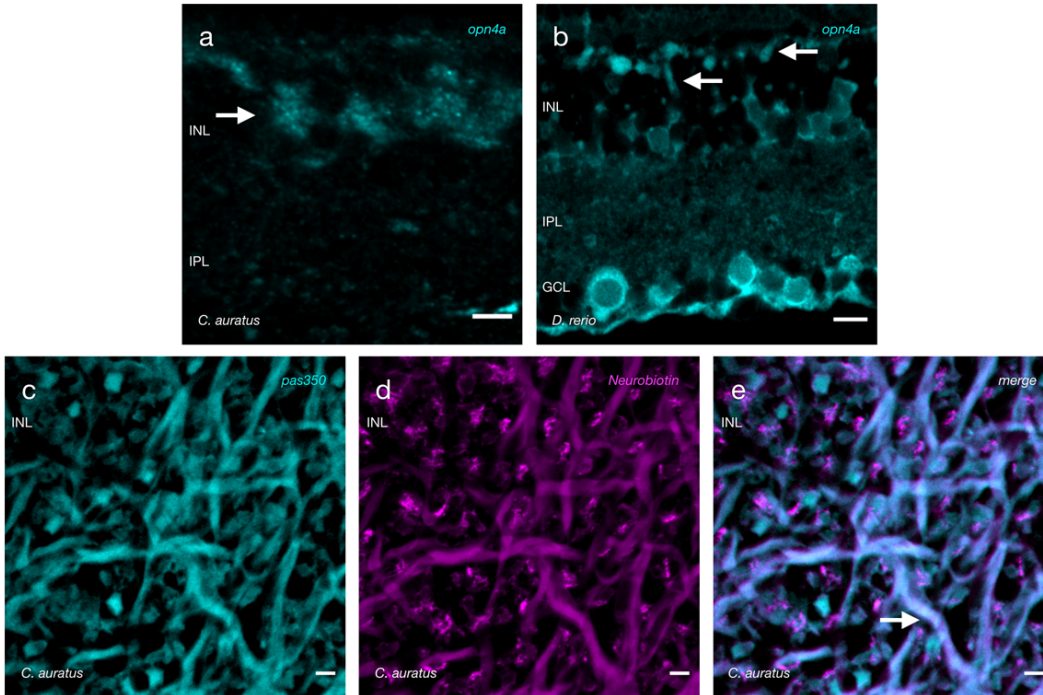
Figure A.8. Horizontal cells are melanopsin-immunoreactive.

A) Presumptive H1-type HATs are labelled by pas350 (pictured) and opn4. TH punctate labelling is seen at the level of the OPL. B) Zebrafish labelling by opn4a shows signal in presumptive horizontal cells and possible axon terminals. C-E) Presumptive H1-type HATs co-label for pas350 when injected with neurobiotin. Bars = 10 μm.

A.5 References

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